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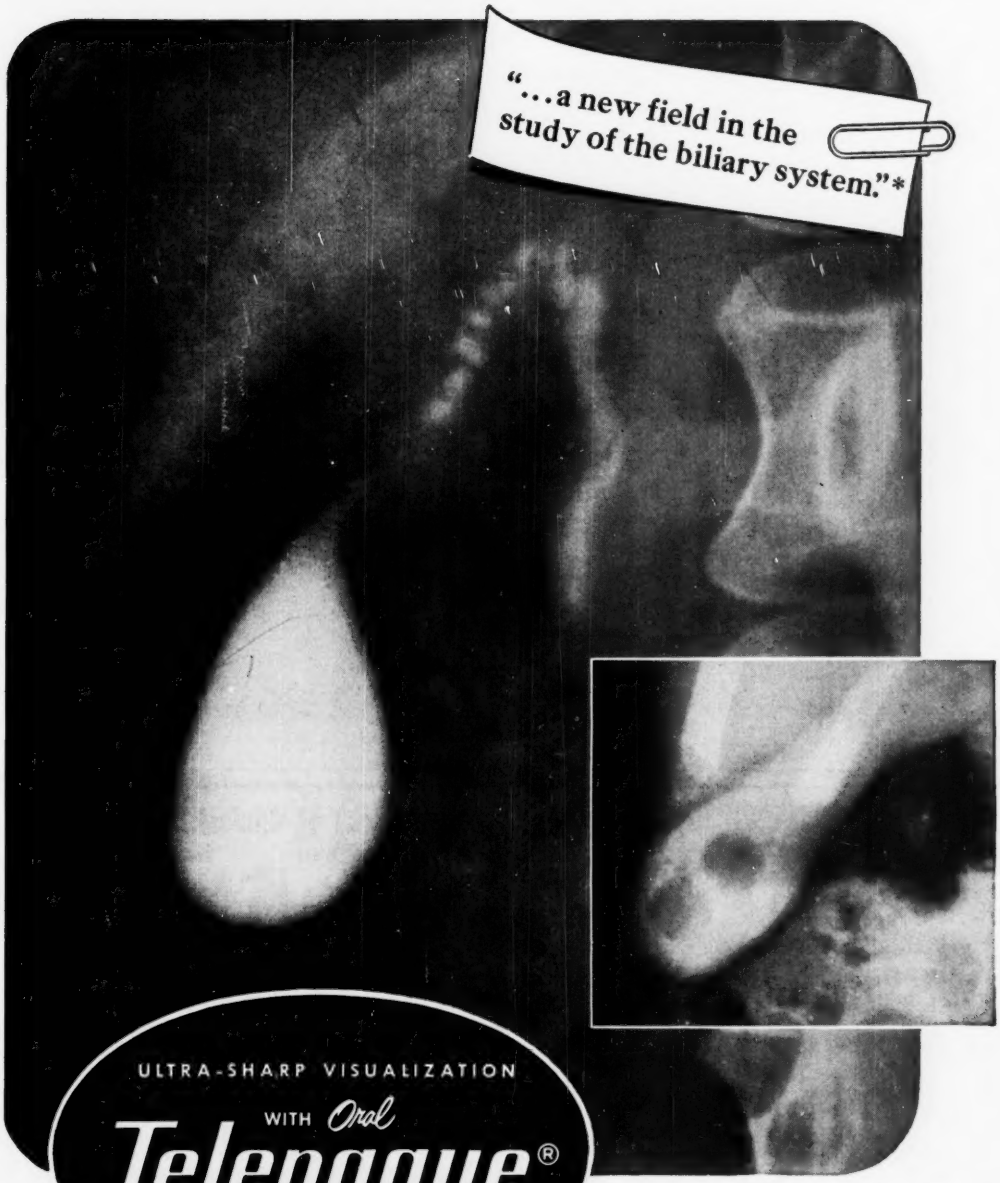

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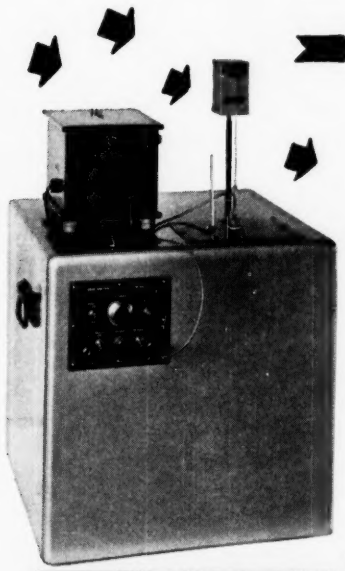

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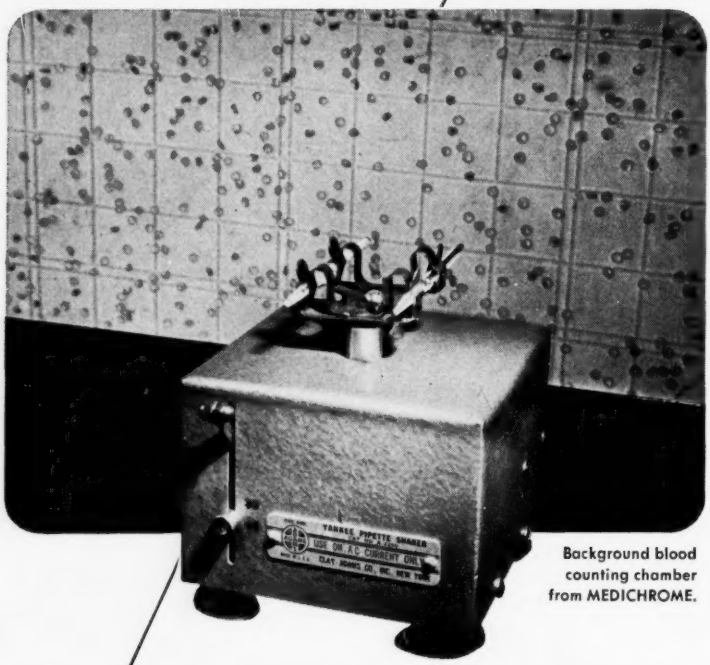

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References
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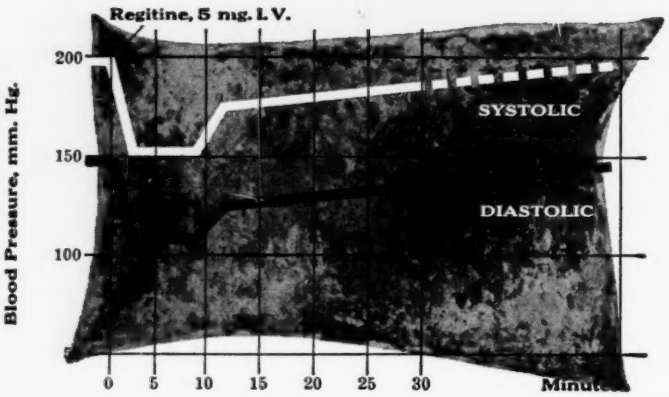

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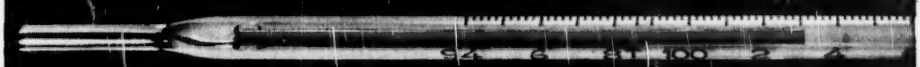

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THE RATE OF UPTAKE OF NITROUS OXIDE IN MAN [1]

By J. W. SEVERINGHAUS [2]

(From the Department of Anesthesia, Hospital of the University of Pennsylvania, and Harrison Department of Surgical Research, University of Pennsylvania, Philadelphia, Pa.)

(Submitted for publication January 18, 1954; accepted May 14, 1954)

During inhalational anesthesia, an inert gas or vapor diffuses across the pulmonary membrane, dissolves in the blood and is carried by the circulation to all of the tissues of the body. The rate at which an anesthetic dissolves from the gaseous phase in the alveoli into the pulmonary capillary blood is termed the *rate of uptake* of that gas. The uptake of any inert gas continues for many hours at a slowly decreasing rate until the entire body is saturated with the gas at the inspired tension. For example, Behnke, studying nitrogen gas exchange, found that about 5 hours are required to reach 90 per cent of complete saturation at the inspired gas tension (1).

This slow process of body saturation should be differentiated from the process of saturation of the arterial blood, to which the depth of anesthesia is closely related. A normal denitrogenated subject, beginning to breathe air, requires about 3 minutes to reach 90 per cent of complete arterial saturation at the inspired nitrogen tension. If he breathes nitrous oxide, Kety showed that about 20 minutes are required to reach 90 per cent of complete arterial saturation at the inspired nitrous oxide tension (2). Both arterial and body saturation occur more rapidly with a relatively insoluble gas such as nitrogen, than with the soluble anesthetics, since large quantities of the soluble gases are carried away from the lung by the pulmonary blood, delaying the rise of alveolar gas tension.

As the anesthetic gas is taken up in solution in the blood, the resulting decrease in gas volume in a breathing reservoir can be measured by means of a spirometer, just as oxygen consumption is measured in the determination of basal metabolic rate. The method herein reported permits the determination of the volume rate of uptake of the anesthetic gas (in this case nitrous oxide), and oxygen, while maintaining a constant inspired tension of both gases.

The rate of uptake of an inert gas is of interest for the following reasons:

1) Kety and others have attempted to predict mathematically the rate at which soluble gases should be taken up by the body, but the only published experimental data were those in which the relatively insoluble gases nitrogen and helium were used (1, 3, 4). Kety's theoretical uptake curves, designed primarily to predict the course of arterial saturation, are not applicable to body saturation.

2) Behnke (1), Jones (3), and Stevens, Ryder, Ferris, and Inatome (4) independently have studied the nitrogen elimination rate on subjects breathing oxygen over long periods of time, and have concluded that the uptake or elimination rates of other inert gases can be predicted from their data, with certain corrections. Their predictions show a reasonably good correlation with the curves of uptake of nitrous oxide determined experimentally in the present report.

3) It had often been assumed that after 20 to 30 minutes of anesthesia, *all* the body tissues were nearly in equilibrium with the inspired tension of anesthetic gas. Foldes, Ceravolo, and Carpenter (5) describing a technique of nitrous oxide administration with low flow rates, stated that "after a comparatively short period no more nitrous oxide is removed from the administered gas mixture." The observations presented here indicate that nitrous oxide continues to be taken up for at least several hours.

METHOD

Six adult surgical patients without evidence of cardiac or pulmonary disease, were studied during prolonged surgical procedures. After topical application of cocaine, a cuffed endotracheal tube was inserted, through a previously prepared tracheotomy in three cases, and per-orally in the other three. Induction and supplementation of anesthesia were accomplished using intravenous thiopental. The residual nitrogen of the lung was re-

[1] Presented in part at the Federation Meetings, April, 1954.

[2] Present address: National Institutes of Health, Bethesda, Md.

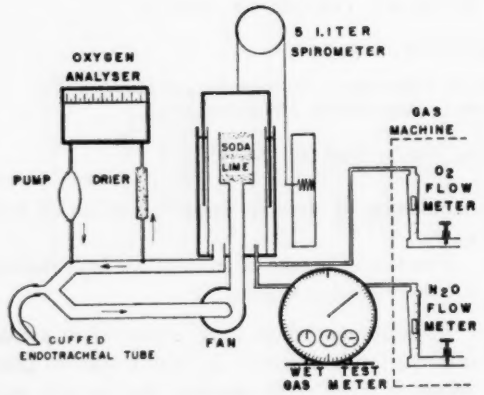

FIG. 1. THE ARRANGEMENT OF APPARATUS USED FOR DETERMINING THE NITROUS OXIDE UPTAKE RATE

An anesthesia gas machine was modified to permit the flow of N_2O to be measured with a wet-test gas meter before mixing it with O_2. The O_2 analyzer pump was a hand bulb. The flow rates of N_2O and O_2 were adjusted to keep the end expiratory spirometer volume constant, and the inspired O_2 concentration at 20 per cent.

placed with oxygen administered in an open circuit (no rebreathing) for at least 10 minutes. The endotracheal tube was then connected to a spirometer containing a circulating fan and CO_2 absorbent (Figure 1). The total

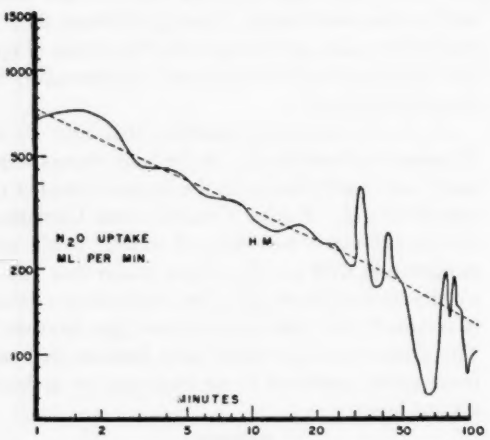

FIG. 2. AN EXAMPLE OF THE OSCILLATIONS IN UPTAKE RATE DUE TO VARIATIONS IN THE INSPIRED CONCENTRATION OF N_2O IN ONE PATIENT DURING 100 MIN. OF ANESTHESIA

On log-log coordinates the curve of uptake rate *vs.* time was most nearly a straight line. This allowed the best estimate of the N_2O uptake which would occur if inspired concentration were held constant, by visually selecting a line to average out the oscillations.

gas capacity of the spirometer and tubing was 12 liters consisting of a 5 liter bell and 7 liter residual volume. It had been filled to 10 liters with 100 per cent nitrous oxide (in 2 cases 90 per cent N_2O, 10 per cent O_2). The respiration of the subject then mixed the lung gas, oxygen, with the spirometer nitrous oxide. Normally at the end of expiration the lungs contain 2 to 3 liters of gas (the functional residual capacity). As this volume of oxygen mixed with 10 liters of N_2O, the O_2 concentration in the spirometer rose, and at the same time, the alveolar O_2 concentration fell. The inspired oxygen tension was measured with a Pauling type oxygen analyzer [3] so arranged that the analyzed gas samples were returned to the system. Nitrous oxide was added to the spirometer through a wet-test gas meter which indicated the total volume of flow. The flow rates of both O_2 and N_2O were controlled with the needle valves and rotameters on a conventional anesthesia machine. The method required that one observer continuously monitor both the inspired O_2 concentration and the end-expiratory spirometer volume. He adjusted the rates of flow of both O_2 and N_2O to hold the inspired O_2 concentration at 20 per cent and the volume constant. If these criteria were achieved, then both O_2 and N_2O were added to the system as rapidly as they were taken up in solution by the body. At one minute intervals, a second observer recorded the total volume of N_2O that had been added through the wet-test gas meter, and the inspired oxygen concentration.

The inspired N_2O concentration was calculated to be 100 per cent minus the inspired O_2 concentration. Actually, there was always a small gradual rise in the N_2 concentration as it diffused outward from the tissues of the body, in one case (R. E.) amounting to 7 per cent after 65 minutes. It follows that both the N_2O concentration and its rate of uptake decreased slowly during the experiment. The resulting inspired N_2O concentration at the end of the experiment was probably between 70 per cent and 75 per cent when the O_2 concentration measured 20 per cent.

It was usually not possible to keep the inflow of nitrous oxide exactly equal to the rate of uptake by the patient. A correction was made in calculating the uptake rate based on the changes in spirometer volume and O_2 concentration from minute to minute.

RESULTS

The rate of uptake of N_2O tended to oscillate (Figure 2) as a result of concentration changes in the alveolar gas. The rate of uptake is determined by the gradient in gas tension between alveolar gas and mixed venous blood. After partial saturation, small changes in alveolar N_2O tension, caused by changes in inspired tension, may produce very large percentage changes in the gradient between alveoli and mixed venous blood tension.

[3] Beckman Instruments, South Pasadena, Calif.

For example, if after one-half hour the tension gradient is 20 mm. Hg, a fall of alveolar N_2O tension of 20 mm. (3 per cent in concentration) would result in temporary equilibrium, and the uptake of N_2O would cease. These concentration changes result from the human inability to rapidly correct gas flow rates, a factor which is considerably reduced by experience.

In Figure 2 the measured rate of uptake of N_2O of one of the six subjects is presented on log-log coordinates; as suggested by Stevens, Ryder, Ferris, and Inatome (4) these coordinates most nearly result in a straight line relationship between rate of uptake and time. A line was selected visually to average out the oscillations in the rate of uptake of N_2O. In Figure 3 the N_2O uptake rates of the six subjects corrected by this graphical method are plotted on semi-logarithmic coordinates. This procedure introduces errors in the reporting of the actual gas uptake, but none are cumulative, so the result is an approximation of the uptake rate which would occur with a constant inspired concentration of N_2O.

The total volume of N_2O taken up in solution in the body of each of the six patients is listed in Table I. This volume is compared with the volume of the gas which would be required to saturate the body at a partial pressure of 570 mm. (inspired concentration 80 per cent), knowing the solubilities at this pressure to be 1,150 ml. per liter of adipose tissue and 360 ml. per liter of watery tissues. The body was assumed to be 20 per cent fat and 72.4 per cent watery tissue (1); these predicted values are therefore only approximations. They are presented to indicate that the large volume of gas absorbed by the patient is reasonably in accord with expectations.

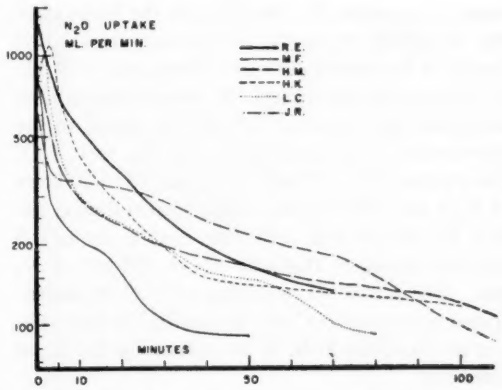

FIG. 3. THE RATE OF UPTAKE OF N_2O BY SIX SUBJECTS DURING ANESTHESIA WITH 80 PER CENT N_2O AND 20 PER CENT O_2.

These curves were obtained from the measured values by the graphical procedure shown in Figure 2.

Oxygen consumption in Table I was roughly measured with the rotameter in four subjects. The reasonable agreement with predicted metabolic rate serves to demonstrate the absence of significant gas leaks during the procedure.

DISCUSSION

There are several sources of error in the determination of the rate of uptake of N_2O. Cumulative errors arise from nitrogen in the inspired gas, and from loss of nitrous oxide through the skin, the wound and rubber tubing. These errors are in opposite directions. Nitrogen, by its presence, lowers the inspired concentration of nitrous oxide, thereby lowering the rate of nitrous oxide uptake; its sources are: 1) the N_2 dissolved in the tissues of the body, amounting to 700 to 1,200 ml., some of which diffuse outward during the experi-

TABLE I

The rate of uptake of nitrous oxide and oxygen

Patient	Age	Sex	Height	Weight	Duration of experiment *minutes*	Predicted total N_2O uptake in liters when saturated	Measured N_2O uptake at end *liters*	O_2 consumption *ml./minute*	
								Predicted	Measured
R. E.	16	M	58	156	67	36.4	21.69	254	
H. K.	48	M	65	100	109	23.3	22.13	190	
L. C.	21	F	64	129	80	30.0	16.5	200	193
M. F.	34	F	65 est	120	50	28.0	7.58	190	190
H. M.	48	M	66	156	108	36.4	19.0	224	165
J. R.	49	M	74	162	155	37.8	29.95	260	203

ment; 2) gaseous N_2 remaining in the lungs after the 10 minute washout. In normal subjects this should be less than 2 per cent of lung gas, or 60 ml; 3) diffusion of atmospheric N_2 inward through the skin and open wounds (1); 4) N_2 added to the spirometer as an impurity in the O_2 and N_2O. Since about 15 to 25 liters of O_2 and 16 to 30 liters of N_2O are added to the closed system during the first 1½ hours, and both may contain up to 0.5 per cent nitrogen, this adds about 250 ml. of N_2 gas. The actual concentration of N_2O in the inspired air could have been determined in each subject as was done in R. E. by measuring the N_2 at intervals.

Loss of N_2O gave falsely high values for uptake rate. The only important source of loss of N_2O is diffusion outward through the skin and the open wound. Orcutt and Waters (6) estimated the loss through the skin of the entire body to be about 7 ml. per min. Wounds probably give rise to a considerable additional loss.

Some error is introduced by the variations of volume and concentration previously mentioned, but these are not cumulative, and tend to average out with time. The graphical method of cancelling out these oscillations is of course an approximation.

The wet-test gas meter is accurate to within 0.5 per cent at any volume. The oxygen analyzer is accurate in the presence of N_2O and at 20 per cent O_2 concentration is readable to 0.5 per cent, accurate to 1.0 per cent.

The prediction of N_2O uptake rate

The process of saturation of the body with a gas is believed to be exactly the reverse of the process of desaturation with the same gas (2). For example, if 5 hours of O_2 breathing are required to remove 90 per cent of the body N_2, then 5 hours of air breathing will be required to replace 90 per cent of the N_2. Furthermore, two gases with identical solubility coefficients will have identical uptake rates. Both Jones and Behnke have suggested that the uptake of any gas might be predicted from the nitrogen elimination rate according to the relative solubilities of the two gases.

Increased solubility of the gas in blood will delay the rise of arterial and alveolar tension, since a larger share of the gas will be removed from the alveoli and carried away by the blood. Fortunately, the time required for arterial saturation with N_2O at the inspired tension has been frequently measured by others (2). In general, 90 per cent saturation will occur in about 20 minutes with normal cardiac output and alveolar minute ventilation.

It is possible to predict from N_2 elimination data the volume of N_2O needed to saturate the entire body, as follows. N_2O is 32 times more soluble than N_2 in blood; it is 20 times more soluble than N_2 in fat. With 20 per cent of the body being fat, the average solubility of N_2O will be 30 times that of N_2. Since in these studies the inspired concentration of N_2O was 80 per cent, this gas saturated the body at the same tension that N_2 had exerted. Therefore, by volume, 30 times more N_2O than N_2 will dissolve in the body at the same concentration.

The prediction of the *rate* of uptake now involves several additional considerations. Tissue perfusion rate (not gas solubility) limits the saturation of the individual non-fat tissues of the body with an inert gas (3). Therefore, when arterial saturation is complete, each tissue (whether fat or non-fat) will reach saturation with N_2O at about the same rate that it becomes desaturated with N_2. This is true of fat only because of the similar fat to plasma partition coefficients for these two gases (5.2 to 1 for N_2 and 3.2 to 1 for N_2O). Gases with a high fat to plasma partition coefficient such as cyclopropane (35 to 1) require a longer time to fully saturate the fat. Therefore, in translating from N_2 data to N_2O predictions, the assumption was made that fat had a similar influence on the two gas exchange rates. Thus, for equal arterial tensions, the rate of uptake of N_2O will also be 30 times the rate of elimination of N_2.

The N_2 elimination rates of volunteers have been published by Jones (3), Stevens, Ryder, Ferris, and Inatome (4) and Behnke and Willmon (1, 7). These data were utilized by multiplying the rate of N_2 elimination by the factor

$$30 \times \frac{\text{arterial nitrous oxide tension}}{\text{inspired nitrous oxide tension}}$$

assuming 70 per cent of inspired tension after 3 minutes and 90 per cent after 20 minutes. These several curves predicting the rate of uptake of N_2O are compared graphically in Figure 4 with the

average N_2O uptake as measured in the six subjects reported herein. The correlation is reasonably good in spite of variations in body size, amount of adipose tissue, cardiac output, pulmonary ventilation, other effects of anesthesia, and the many assumptions made both in the predicted and the measured uptake rates. Ventilation in the anesthetized subjects was undoubtedly less than that in the conscious volunteers studied for N_2 elimination.

The average N_2O uptake of the six subjects when plotted on log-log coordinates (Figure 4) is nearly a straight line. An approximate formula fitting this straight line is N_2O uptake rate $= 1{,}000\ t^{-0.5}$. Stevens provides formulae for several subjects, which, converted by the factor 30 for comparison with N_2O, for a 60 Kg. body weight would give, with wide variations, N_2O uptake rate $= 1{,}800\ t^{-0.6}$.

Behnke's data were graphically differentiated to obtain rate from his published curves of total N_2 elimination. Jones' curves were obtained by differentiation of a 5 term exponential equation.

Also plotted in Figure 4 is a curve of N_2O uptake as predicted by use of Kety's theoretical equations for arterial and mixed venous concentration *vs.* time. The similarity of slope during the first 30 minutes is in accord with the derivation of this equation intended to predict the course of arterial saturation.[4]

The rough agreement between the experimentally determined N_2O uptake rate and the predicted N_2O uptake rate supports the thesis that, as an approximation, the N_2O uptake rate is 30 times the N_2 elimination rate in ml. per min. throughout the process of body saturation.

The significance of these observations in anesthesia

Nitrous oxide is commonly used in a semi-closed or partial rebreathing system to provide a pleasant induction to ether anesthesia. After induction has been completed, the flow of N_2O is stopped and ether is administered in a closed system with CO_2 absorption. It is generally recognized that, during induction, the inspired concentration of O_2 is lower than the concentration of O_2 in the administered mixture because of the absorption of O_2 by the pulmonary blood. However, the high rate of uptake of N_2O tends partially to cancel out the effect of oxygen absorption on the inspired concentration. If the two gases were taken up in proportional amounts, which they are during the first 1 to 3 minutes, no reduction in oxygen concentration would occur. It is therefore reasonable to use a slightly lower O_2 proportion in the administered mixture during induction than during maintenance of anesthesia with N_2O.

A common clinical observation is the tendency for the depth of anesthesia to lessen if the flow of N_2O is stopped, as when used for induction of ether anesthesia. The explanation for this occurrence is as follows: After the flow of N_2O has ceased, the gas continues to be taken up in solution

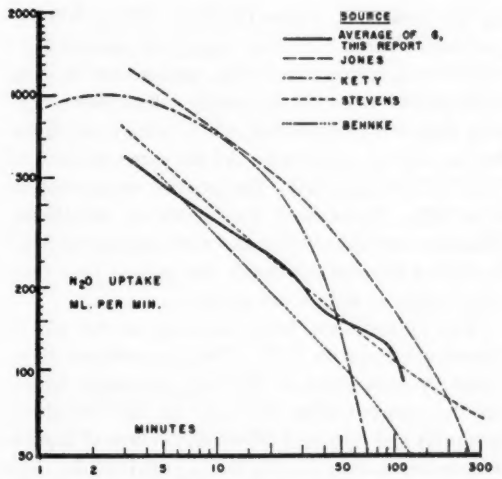

FIG. 4. COMPARISON OF THE AVERAGE OF THE CURVES OF N_2O UPTAKE OF SIX PATIENTS, DISREGARDING BODY SIZE, WITH THE RATE OF UPTAKE OF N_2O AS PREDICTED BY VARIOUS METHODS

The curve from Kety's theoretical equation was predicted by assuming the following physiological parameters: cardiac output 6 L. per m, alveolar minute ventilation 3 L. per m, functional residual capacity 3 L., wt. 70 Kg. The curves from Jones, Behnke and Stevens were obtained by multiplying the N_2 elimination rate curves by the factor $\left(30 \times \frac{\text{arterial } N_2O \text{ tension}}{\text{inspired } N_2O \text{ tension}}\right)$.

[4] Using Kety's symbols, the equation for the rate of uptake of an inert gas, derived from his equation 55, is as follows:

$$\text{Rate of uptake} = \frac{dC_v}{dt} \cdot V_t$$
$$= C_I \cdot [A_1 M_A - V_A k_1] \cdot [e^{-k_1 t} - e^{-k_2 t}].$$

by the patient at a rate of about 200 to 500 ml. per min. This depletes the supply of gaseous N_2O in the lung and reservoir bag, and lowers its concentration. Also, O_2 is usually added more rapidly than it is metabolized, and is used to refill the bag as N_2O is absorbed. As the concentration of N_2O in the lung falls, the arterial concentration also falls, diminishing the depth of anesthesia. Since the amount of ether absorbed during the N_2O induction is relatively small, the patient may then show signs of lighter anesthesia.

This information has a bearing on the use of closed systems with N_2O. The gas continues to be taken up in solution in the body for many hours, so the concentration of N_2O in the breathing reservoir and lung will fall when the flow of N_2O is stopped even after several hours, provided the oxygen flow is at least sufficient to meet metabolic needs. This precludes the maintenance of an even depth of anesthesia with N_2O in a closed system unless the O_2 concentration is periodically measured. Several reports describe the use in semi-closed systems of low flow rates of both O_2 and N_2O which have been shown to result in desirable O_2 concentrations (5, 8). The resulting gas concentrations will depend somewhat on the uptake of N_2O and will vary, accordingly, with the duration of anesthesia.

The time required for elimination of N_2O from the body during the recovery period is similar to that required for saturation with the gas; it follows that the body tissues as a whole require about 5 hours to lose 90 per cent of their N_2O. Arterial blood likewise loses 70 per cent of its N_2O in 3 minutes, 90 per cent in 20 minutes.

Fink, Carpenter, and Holaday (9) have demonstrated that this large volume of N_2O being eliminated during the first few minutes of air breathing results in a lowered alveolar oxygen tension, which may be enough to produce arterial unsaturation with usually adequate ventilation.

The metabolic rate during anesthesia, as reflected in O_2 consumption, can be measured by the method used herein for N_2O uptake, if O_2 is added through a wet-test gas meter. At least 20 minutes should be allowed to average out the previously mentioned oscillations in concentration and reservoir volume. Although this was not done in the present study, the setting of the O_2 flowmeter was recorded in four of the six patients. Table I lists the average O_2 consumption during the entire procedure calculated from this data, along with the O_2 consumption predicted for each subject under basal conditions according to the DuBois tables.

SUMMARY

The volume rate of uptake of N_2O by the body was measured during surgical anesthesia. After 90 minutes of inhalation of an 80 per cent N_2O — 20 per cent O_2 mixture, the body is still absorbing about 100 ml. of N_2O per minute from the gaseous phase in the lungs. Seven and one-half to 30 liters of N_2O are taken up in solution in the body during 1 to 2½ hours of anesthesia.

The rate of uptake of N_2O during at least the first 2 hours of anesthesia is about 30 times the volume rate of elimination of nitrogen as reported by others (30 is the ratio of solubility of the two gases). This evidence supports the suggestion that the approximate uptake rate of other inert gases can be predicted from data on nitrogen elimination.

The average rate of uptake of N_2O in six subjects was described approximately by the equation: Rate = $1{,}000\ t^{-0.5}$ ml. per min.

The experimental findings were discussed in relation to several clinical problems in anesthesia.

The method can be used to determine the oxygen consumption as an index of the metabolic rate during anesthesia with gases or vapors.

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STUDIES ON STAPHYLOCOCCI FROM HOSPITAL PATIENTS. I. PREDOMINANCE OF STRAINS OF GROUP III PHAGE PATTERNS WHICH ARE RESISTANT TO MULTIPLE ANTIBIOTICS [1, 2]

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Studies from widely separated geographical areas (1–5) have revealed that a great majority of staphylococci from hospital patients which are resistant to penicillin are lysed by group III staphylococcal bacteriophages. Furthermore, in two of these studies (2, 5), it was found that strains of staphylococci found to be resistant to tetracyclines were also resistant to penicillin and lysed by group III phages. Since group III bacteriophages were named because they lysed staphylococci of Cowan's serologic group III, it appeared that one biologic subdivision of human pathogenic staphylococci was the chief source of strains from hospital patients in several areas which were resistant to antibiotics.

At Bellevue Hospital in 1953–54, these observations were confirmed, and it was additionally found here that resistance to streptomycin was also a characteristic of this group of staphylococci.

Evidence was obtained that this phenomenon probably resulted from the widespread use of antibiotics in hospitals, when it was found that staphylococci isolated at a nearby hospital in the period 1932–48, at a time when, for the most part, no antibiotics were available, exhibited few of the characteristics described above. Moreover, based on a study of strains isolated from patients soon after admission to Bellevue Hospital in 1953–54, it was determined that multiple drug resistance and lysis by group III phages were not important characteristics of staphylococci carried by non-hospitalized persons, to whom antibiotics are less frequently administered. Finally, it was found that the rate at which newly hospitalized patients acquired staphylococci characteristic of the hospital was correlated with the kind of antimicrobial treatment they received.

The report which follows consists of a description of these studies.

PLAN OF STUDY

Clinical material

Cultures for staphylococci were made three times weekly of the nose, throat, and rectum of a large number of patients at Bellevue Hospital with non-staphylococcal illness. From about one-half of them, a total of 80 patients, were obtained cultures of staphylococci (*Micrococcus pyogenes* var. *aureus*). Sixty-seven of the patients who had infectious diseases, principally pneumococcal pneumonia, were treated as follows: 30 were given penicillin alone or in combination with streptomycin; 25 received chlortetracycline or oxytetracycline; and 12 got other combinations of antibiotics. Thirteen patients with non-infectious illnesses, including cases of duodenal ulcer, cirrhosis of the liver, hepatitis, and heart disease, received no antimicrobial therapy. From these 80 patients were obtained 414 strains of staphylococci.

Six additional patients with serious staphylococcal infection were included in the study, and from them 102 strains of staphylococci were obtained, 24 of which were cultured from blood or localized staphylococcal lesions. The characteristics of strains from these patients were generally similar to those from non-staphylococcal cases.

Fifty-five strains of staphylococci (*Micrococcus pyogenes* var. *aureus*) which had been collected at Postgraduate Hospital in New York [3] in the period 1932–48 were also available for this investigation. Thirty-nine strains were cultured from blood and the remainder were from other sites of staphylococcal infection.

[1] Presented in part before the Antibiotics Symposium of the Food and Drug Administration, Department of Health, Education and Welfare, Washington, D. C., October, 1953.

[2] This investigation was supported by grants from Charles Pfizer and Company, Brooklyn, New York; Parke, Davis and Company, Detroit, Michigan; and by a research grant (E-330) from the National Institutes of Health, Public Health Service, Bethesda, Maryland.

[3] These strains were collected by the late Dr. Ward MacNeil and Miss Anne Blevins, and were preserved by Dr. John Blair, Hospital for Joint Diseases, New York City, who has kindly provided subcultures for use in this investigation.

TABLE I

Susceptibility of staphylococcus aureus to antibiotics

1932–48 Postgraduate Hospital Collection 55 strains
1953–54 Bellevue Hospital Collection 516 strains

	Per cent sensitive		Per cent mod. sens.		Per cent mod. resist.		Per cent resistant		Per cent highly resist.	
	Postgrad.	Bell.	Postgrad.	Bell.	Postgrad.	Bell.	Postgrad.	Bell.	Postgrad.	Bell.
Mcg./ml.	0.1		1.0		10		25		>25	
Penicillin	78	14.9	4	0.7	2	7	9	5.4	7	72.2
Mcg./ml.	3		6		25		50		>50	
Chlortetracycline	100	32.8	—	0.8	—	0.7	—	1	—	65
Oxytetracycline	100	34	—	0	—	0.4	—	0	—	65.5
Streptomycin	4	0.7	36	10.5	56	19.4	—	2.7	4	66.7
Chloramphenicol	2	4.5	9	37.8	82	31	4	17.4	4	9.1
Mcg./ml.	0.8		1.6		6		25		>25	
Erythromycin	89	97	11	0.7	—	0.3	—	0.7	—	1

Microbiologic procedures

Isolation of Staphylococcus aureus. Nose, throat, and rectal swabs were streaked on plates of trypticase soy agar containing 0.25 phenyl ethyl alcohol, and the rectal swab was later incubated in a tube of Brewer's thioglycollate broth. A subculture was made of a representative colony of staphylococcus from each culture, which fermented mannitol, hemolyzed equine or human red cells, and coagulated citrated human plasma, and was preserved for the study. In the event that no colonies resembling staphylococci were found on plates inoculated with rectal swabs, the thioglycollate broth was examined, and very exceptionally it yielded strains of staphylococci with the above characteristics.

Susceptibility to antibiotics. The technic described by Jackson and Finland (6), employing agar plate dilutions, was used to test the susceptibility of the strains to antibiotics.

Penicillinase production test. The procedure described by Gots and modified by Haight and Finland (7) was used to test for penicillinase production of 398 of the strains of staphylococcus in this study. There was a close correlation between resistance to penicillin and production of the enzyme by strains of staphylococcus. Since these findings correspond closely to observations by many others, no further description of the results will be made.

Determination of patterns of lysis of staphylococci by bacteriophage (phage typing). The methods of phage typing described by Williams and Rippon (8) and by Blair and Carr (9) were used in this study. The procedure consisted of exposing strains of staphylococci to be identified (typed) to a series of staphylococcal bacteriophages in certain specified dilutions. If a strain was not lysed by any of the diluted bacteriophage suspensions, it was retested with undiluted bacteriophage. In the present investigation, 54 per cent of 516 strains exhibited patterns of lysis when exposed to bacteriophages in specified dilutions, and an additional 41 per cent were lysed when retested with undiluted phage. Only 5 per cent of strains were not lysed (untypable) in the study.

Twenty-five different staphylococcal bacteriophages were used in the investigation.[4] They were divided into three series according to their capacity to lyse strains of staphylococci of Cowan's serologic groups, as follows: Cowan's staphylococcal group I, phages 52, 52a, 29, 29a, 31, 44, 44a; Cowan's group II, phages 3a, 3b, 3c, 51, 39, 523; Cowan's group III, phages 6, 7, 42b, 42d, 42e, 47, 47b, 47c, VA4; and unclassified, 42c, 47a, and 142.

Staphylococcal strains were designated as members of phage group I, II, or III, according to their susceptibility to lysis by the respective groups of bacteriophages. There was a small amount of overlapping of reactions among the three groups, especially when undiluted phages were used, but there was rarely any difficulty in picking out the phage groups with the predominating lytic reactions.

RESULTS

Comparison of antimicrobial susceptibility and phage patterns of staphylococci collected at Postgraduate Hospital in the period 1932 to 1948, with strains isolated at Bellevue Hospital in the period 1953 and 1954

Susceptibility to antimicrobial agents (Table I). The Postgraduate Hospital collection of 55 strains of staphylococci were all found to be highly susceptible to the tetracyclines and erythromycin. Approximately three-fourths were highly susceptible to penicillin, while chloramphenicol and streptomycin inhibited most strains in concentrations in the intermediate range of susceptibility.

[4] The bacteriophages and the propagating strains of staphylococci were kindly provided by Dr. John Blair.

In contrast, almost three-fourths of the Bellevue Hospital strains of staphylococci were highly resistant to penicillin, and two-thirds were highly resistant to the tetracyclines and streptomycin. There was little difference in susceptibility to chloramphenicol and erythromycin from the 1932–48 collection, except for the presence of a few strains which had been observed to become resistant to the latter drug after exposure to it in treatment.

Correlations of resistance and susceptibility to the various antibiotics among the Bellevue Hospital strains revealed (Table II) that about two-thirds of the 516 strains were resistant to the four antibiotics, penicillin, chlortetracycline, oxytetracycline, and streptomycin. Among the remaining strains, the only recognizable pattern was a tendency toward association of resistance to penicillin and streptomycin.

TABLE II

Correlations among grades of susceptibility to four antibiotics

		No. strains	Per cent
Resistant* to	Penicillin Streptomycin Tetracycline derivatives	338	65.5
Resistant to Sensitive† to	Penicillin Streptomycin Tetracycline derivatives	75	14.5
Resistant to Sensitive to	Penicillin Tetracycline derivatives Streptomycin	1	.2
Resistant to Sensitive to	Penicillin Streptomycin Tetracycline derivatives	25	4.9
Sensitive to Resistant to	Penicillin Streptomycin Tetracycline derivatives	3	.6
Sensitive to Resistant to	Penicillin Tetracycline derivatives Streptomycin	43	8.3
Sensitive to Resistant to	Penicillin Streptomycin Tetracycline derivatives	1	.2
Sensitive to	Penicillin Streptomycin Tetracycline derivatives	30	5.8
Total		516	100

* Resistant: Penicillin—1 to >25 mcg. per ml.; chlor- and oxytetracycline, streptomycin not inhibited by less than 25 mcg. per ml.

† Sensitive: Penicillin—0.1 mcg. per ml.; chlor- and oxytetracycline, streptomycin—6 mcg. per ml. or less.

Patterns of lysis of staphylococci by bacteriophages. In Table III is shown the bacteriophage patterns (phage types) of the staphylococci collected in the interval 1932 to 1948, classified according to phage grouping I, II, or III. The per cent of strains in each group resistant to penicillin, the tetracyclines, and streptomycin is indicated. Only 20 per cent of the 55 strains were of phage group III patterns, yet more than one-half of all strains resistant to penicillin were in this group. Furthermore, as a point to be discussed later, it was found that five of these latter strains were isolated after penicillin was generally in use in hospitals.

Among the 516 strains from Bellevue Hospital (Table IV), three-fourths were in phage group III and, of these, 89 per cent were resistant to penicillin and 82 per cent were resistant to the tetracyclines and streptomycin. Consequently, more than 90 per cent of strains of staphylococcus resistant to penicillin, the tetracyclines, and streptomycin, singly or collectively, were of group III phage patterns.

Comparison of the rate of appearance in patients during hospitalization of staphylococci of group III phage patterns resistant to penicillin, the tetracyclines, and streptomycin

The studies just described showed that strains of group III phage patterns resistant to multiple antibiotics constituted a large majority of a large sample of staphylococci from patients at Bellevue Hospital in 1953 and 1954. In studies to follow, it will be shown that a negligible number of such strains were present in cultures taken during the first three hospital days (strains presumed to have been brought to the hospital by the patients).

On the basis of these observations, it was suggested that staphylococci of group III phage patterns resistant to multiple antibiotics which appeared in patients during hospitalization were acquired from sources within the hospital, and they

TABLE III

Phage patterns and antibiotic susceptibility of 55 strains of staphylococcus aureus Postgraduate Hospital Collection 1932–1948 *

No. strains	Per cent	Phage pattern	Penicillin resistance Range 1.0 25 mcg./ml. No. strains	Per cent
		Group III		
8		6 7 42b 47 47c VA4 42e+†	5 (1940, 1946, 3—1948)	
2		VA4	1 (1948)	
1		42c 42d		
11	20		6	54
3	5	Untypable	1 (1944)	33
		Group I		
12		52a 52+	2 (1938, 1948)	
5		29 31+	1 (1940)	
12		44a 42e‡	1 (1938)	
29	53		4	14
		Group II		
6		3a 3b 3c+	1 (1948)	
3		39		
1		523		
10	18		1	10
		Unclassified		
2		42b 44a 42e 47c+		
2	4			
55	100		12	22

* All strains were inhibited by 3 mcg./ml. of chlortetracycline and oxytetracycline, and those not otherwise indicated were inhibited by 0.1 mcg./ml. of penicillin.
† (+) indicates additional weak lytic reactions were present.
‡ Williams and Rippon state that 42e, 7, 47c may occur with group I phages.

are subsequently referred to as "hospital staphylococci." Furthermore, it was found that important differences in the rate at which these strains were acquired by patients could be correlated with the antimicrobial treatment which they received.

Patients treated with tetracyclines (Figure 1a) acquired "hospital staphylococci" most rapidly. From a value of 16.6 per cent on admission (1 to 3 days), the percentage increased to 90 per cent by the 4- to 5-day period, and thereafter increased to 100 per cent during the 12- to 13- and 14- to 15-day periods. Treatment with penicillin (Figure 1b) was associated with a later and lesser rise in the per cent of "hospital staphylococci" reaching 29 per cent in the 6- to 7-day period and 65 per cent in the 8- to 9-day period. During the following six days, some further increase in the per cent of "hospital staphylococci" was noted.

The group of patients who received no antimicrobial therapy (Figure 1c) showed the lowest rate of acquisition of "hospital staphylococci." During the first three hospital days no "hospital staphylococci" were recovered, and subsequently there were only sporadic elevations in the percentage, ranging from 20 to 43, all values far below the per cent of such strains isolated from antibiotic treated patients.

The per cent of strains in each therapy group resistant to penicillin and the tetracyclines are also shown in Table V and Figure 1 a, b, c. These data reveal that practically all strains resistant to the tetracyclines were in the category of "hospital staphylococci," and, based on the low per cent of these strains isolated in the first three hospital days, it appears that the non-hospital population

TABLE IV

Phage patterns and antibiotic resistance of 516 strains of Staphylococcus aureus Bellevue Hospital Collection 1953–1954

No. strains	Per cent	Phage patterns	Per cent resistant: Oxy- and chlortetracycline *50 mcg./ml.*	Penicillin *25 mcg./ml.*	Streptomycin *50 mcg./ml.*
		Group III			
58		6 47 VA4			
127		47			
10		6 47			
11		47 VA4			
1		6 VA4			
61		VA4			
22		47 47c VA4	82	89	82
32		7			
12		47 3c			
28		42b 47c+			
4		47b 47c+			
10		6 47 42b 47c 47b 42e+			
9		42b 47c 42d 42e+			
1		42d			
386	74.8				
30	5.8	Untypable	33	40	43
		Group I			
32		52a 52+			
2		523 44+			
17		44a 44+	9	20	20
11		44a 42e+			
24		29 31+			
87	17				
		Group II			
13	2.4	3b 3c+	0	0	8
516	100		68	76	69

may not carry many staphylococci resistant to the tetracyclines. In contrast, a majority of staphylococci resistant to penicillin isolated during the first three hospital days were not "hospital staphylococci," and were found to consist principally of phage group I strains. Since about one-half of all admission strains were penicillin resistant, it appeared that a considerable reservoir of staphylococci of phage group I, which were resistant to penicillin, existed in the general population. Many of these penicillin resistant strains which were not classifiable as "hospital staphylococci" continued to be carried by patients during hospitalization who received penicillin or no treatment, but among patients treated with tetracyclines they were replaced by "hospital staphylococci."

DISCUSSION

The foregoing studies have revealed that approximately two-thirds of a large number of strains of staphylococci principally obtained from cultures of the nose, throat, and rectum of staphylococcal carriers at Bellevue Hospital in 1953–54, were highly resistant to penicillin, the tetracyclines, and streptomycin. Moreover, more than 90 per cent of these staphylococci which were resistant to multiple antibiotics were of group III phage patterns.

In marked contrast, among 55 strains of staphylococci collected in the period 1932–48 at Postgraduate Hospital, all were susceptible to the tetracyclines, most were susceptible to penicillin, and the majority were inhibited in the intermediate

range of susceptibility by streptomycin. It is considered that the susceptibility to streptomycin and to the tetracyclines represents about the usual behavior of these organisms with this agent. The small per cent of these staphylococcus strains showing resistance to penicillin, nevertheless, was higher than would have been expected. More than half of these few penicillin resistant staphylococci were of phage group III, and most of them had been isolated after probable exposure to penicillin. Thus it appeared that the correlation between drug resistance of staphylococcal strains and lysis by group III phages, so evident in the 1953–54 collection, was also present to a degree among the strains of the 1932–48 collection, probably as a result of exposure to penicillin.

Other studies revealed that strains of staphylococci of group III phage patterns resistant to several antibiotics were found infrequently in patients during the first three hospital days (*i.e.*, staphylococci presumed to have been brought to the hospital), in contrast to their high predominance in the hospital population in general. Furthermore, it was found that the rate of acquisition of "hospital staphylococci" was greatest among patients treated with tetracyclines, second among patients treated with penicillin, and least among patients who received no antimicrobial therapy.

These events may be tentatively explained in the following way. It was found that a large majority of staphylococci isolated on admission to the hospital were susceptible to tetracyclines, while only about one-half were susceptible to penicillin. Within the hospital, however, was a large reservoir of staphylococci resistant to both tetracyclines and penicillin. It appeared that the large proportion of staphylococci carried on admission which were susceptible to tetracyclines were eliminated by treatment with these agents, and were subsequently replaced by resistant strains from the reservoir in the hospital. A similar but less extensive change apparently took place with respect to penicillin,

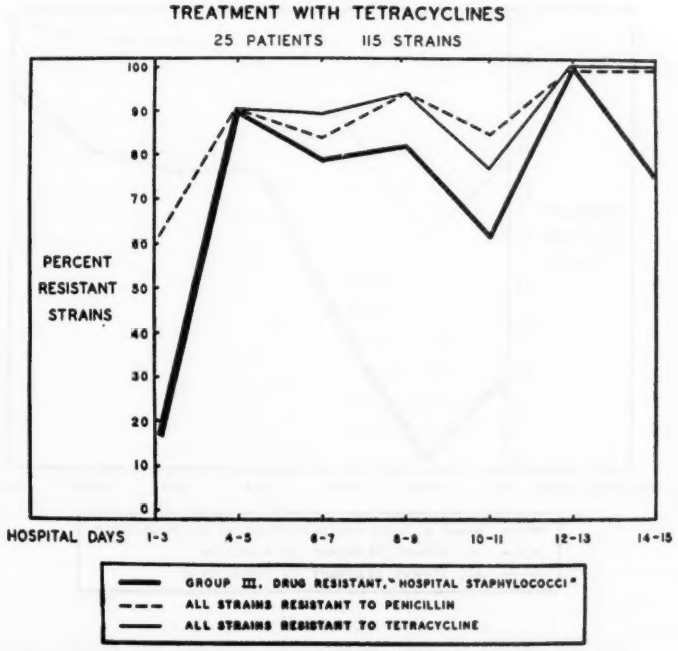

FIGURE 1a

The per cent of staphylococci of group III phage patterns resistant to chlortetracycline, oxytetracycline, penicillin, and streptomycin ("hospital staphylococci") increased from a negligible percentage on admission to a large majority by the 4- to 5-day period, and thereafter remained high.

since only one-half of the staphylococci carried at admission were susceptible to this agent. Among patients receiving no antimicrobial treatment, there was no impetus toward elimination of strains carried on admission, whether susceptible or resistant, so that there was virtually no replacement phenomenon, and acquisition of "hospital staphylococci" was very gradual.

A paradoxical phenomenon observed in these studies was the finding that resistance to penicillin increased significantly more rapidly among strains from patients exposed to tetracyclines in treatment than among strains from patients treated with penicillin. This apparently occurred because of the fact that staphylococci resistant to tetracyclines were also resistant to penicillin, and the higher rate of increase in resistance to tetracyclines was necessarily associated with a similar increase in resistance to penicillin.

The observations which have been presented in this report raise the fundamental question as to why a particular biologic subdivision of human pathogenic staphylococci has become the major source of strains resistant to antibiotics among hospital patients in several areas. Resistance of staphylococci to antibiotics is by no means a unique property of staphylococci of phage group III, and in the present investigation a large proportion of the approximately 60 per cent of strains resistant to penicillin cultured from patients soon after admission were of group I phage patterns. Although the coincident occurrence of the great majority of staphylococci resistant to other antibiotics in phage group III may be indicative of some special property of these staphylococci, it also follows very logically that, if group III strains predominated in an institution as a result of the use of penicillin,

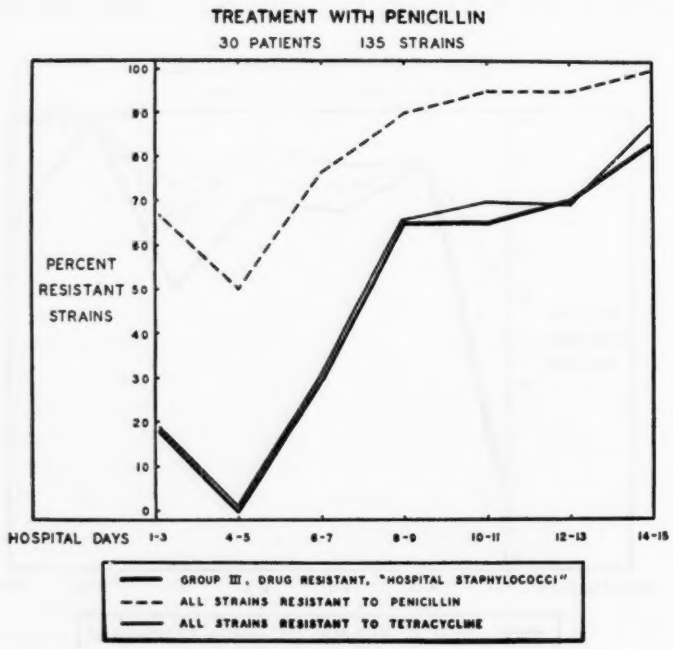

FIGURE 1b

The per cent of "hospital staphylococci" increased less rapidly among strains from patients treated with penicillin than among strains from patients treated with tetracyclines. The per cent of strains resistant to penicillin (including those classifiable as "hospital staphylococci") increased less rapidly among the patients who were treated with penicillin than among those strains isolated from patients treated with tetracyclines.

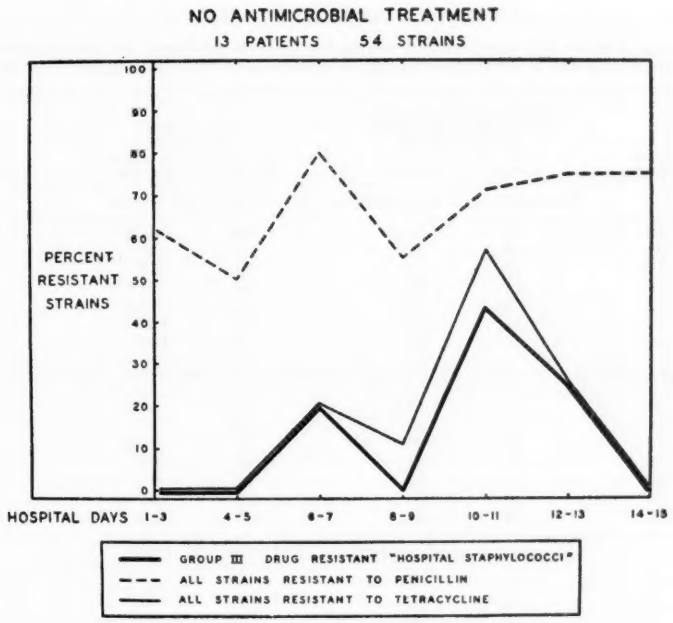

FIGURE 1c

There was only a moderate rise in the per cent of "hospital staphylococci" among strains isolated from patients receiving no antimicrobial therapy.

resistance to other antibiotics introduced later would be greatest among these same strains.

By this line of reasoning one is led to consider the possibility that concentration of staphylococci resistant to antibiotics in phage group III may result from an effect of penicillin which is peculiar to its use in hospitals. Possible characteristics of the administration of penicillin in hospitals which may be important in this regard are the great frequency of its use and the high doses which are given to individual patients. This explanation would not exclude the possibility that there is some property of staphylococci, present to a greater degree among strains of group III phage patterns, which causes their selection in this situation.

SUMMARY

In a study of 516 strains of Staphylococcus aureus (*Micrococcus pyogenes* var. *aureus*), principally cultured from the nose, throat, and rectum of staphylococcal carriers at Bellevue Hospital in 1953 and 1954, it was found that 65 per cent were resistant to the four antibiotics, chlortetracycline, oxytetracycline, streptomycin, and penicillin. More than 90 per cent of these drug-resistant strains were of phage group III patterns. The strains of staphylococci were of intermediate degrees of susceptibility to chloramphenicol, and nearly all were highly susceptible to erythromycin.

In contrast, 55 similar strains collected in the period 1932 to 1938 were to a large extent susceptible to penicillin, and were all inhibited by low concentrations of the tetracyclines and erythromycin. They were of intermediate degrees of susceptibility to streptomycin and chloramphenicol, and only 20 per cent were in phage group III.

Few staphylococci of group III phage patterns resistant to multiple antibiotics were isolated on admission from staphylococcal carriers at Bellevue Hospital, but thereafter, among such patients who were treated with tetracyclines, these "hospital staphylococci" rapidly replaced other strains of staphylococci. Cultures from patients treated with penicillin underwent a similar but significantly

TABLE V

Per cent of staphylococci of group III phage patterns resistant to multiple antibiotics, per cent resistant to penicillin, and per cent resistant to tetracyclines by day of isolation at Bellevue Hospital in 1953–54

Day of isolation	Total strains	Group III strains resistant to penicillin, tetracyclines, and streptomycin		Strains resistant to penicillin		Strains resistant to tetracyclines	
		Number	Per cent	Number	Per cent	Number	Per cent
Treatment with tetracyclines—25 patients—115 strains							
1–3	26	4	16.6	16	61.5	4	16.6
4–5	21	19	90	19	90	19	90
6–7	19	15	79	16	84	17	89.5
8–9	17	14	82	16	94	16	94
10–11	13	8	61.5	11	84.6	10	77
12–13	11	11	100	11	100	11	100
14–15	8	6	75	8	100	8	100
Treatment with penicillin, alone or with streptomycin—30 patients—135 strains							
1–3	28	5	18.5	18	66.6	5	18.5
4–5	10	0	0	5	55	0	0
6–7	17	5	29.2	13	76.5	5	29.4
8–9	20	13	65	18	90	13	65
10–11	20	13	65	19	95	14	70
12–13	23	16	70	22	95	16	69.4
14–15	17	14	82.4	17	100	15	88.4
No antimicrobial treatment—13 patients—54 strains							
1–3	13	0	0	8	61.5	0	0
4–5	8	0	0	4	50	0	0
6–7	5	1	20	4	80	1	20
8–9	9	0	0	5	55.5	1	11
10–11	7	3	43	5	71.3	4	57
12–13	8	2	25	6	75	2	25
14–15	4	0	0	3	75	0	0

Statistical analysis: (*Chi-square test*)

4- to 5-day period	Penicillin resistance in penicillin treatment group compared to penicillin resistance in tetracycline treatment group	Probability of chance occurrence <0.02
4- to 9-day period	Per cent "hospital staphylococci" in penicillin treatment group compared to tetracycline treatment group; per cent in penicillin treatment group compared to no antimicrobial treatment group	Probability of chance occurrence <0.01

less rapid change, while only a moderate increase in the percentage of the strains of "hospital staphylococci" occurred among patients who received no antibiotics.

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HISTAMINE METABOLISM IN DISEASES OF THE LIVER

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Recent experimental studies in animals indicate that the liver plays a part in the inactivation of histamine in the body, by removing this substance from the blood (1) and by acetylating it to form acetyl or conjugated histamine (2–5). The supposition, therefore, that disorders of hepatic function in man may alter the metabolism of histamine seems reasonable. Some clinical evidence of this is provided by the studies of Chambon and Berthier (6) who found that marked hepatic insufficiency or cirrhosis was often accompanied by high levels of histamine in the blood.

A disturbance of histamine metabolism has been held responsible for the pruritus which sometimes occurs in obstructive jaundice (7). Bile salts have been shown to release histamine from the skin (8) and from the liver (1), and Anrep and Barsoum (9) reported that ligature of the common bile duct in dogs caused a rise in blood histamine which they attributed to release of histamine by retained bile.

Our study was undertaken to determine whether an abnormal metabolism of histamine could be detected in patients with disordered hepatic function by examination of the blood and urine, and whether in patients with obstructive jaundice any changes which might be found could be related to the degree of itching.

METHODS AND MATERIAL

Histamine was estimated in blood by a modification (10) of the method of Barsoum and Gaddum (11). After the trichloracetic acid filtrate had been boiled with hydrochloric acid and dried twice in the presence of alcohol, the dry material was extracted three times, using 10, 5, and 5 ml. aliquots of absolute alcohol, in order to remove potassium (12). The alcohol-insoluble material was separated by centrifugation, and the alcoholic extract then taken to dryness. The dried residue was dissolved in diluted Tyrode's solution, neutralized and then used in the final assay of histamine on a segment of guinea-pig ileum.

Blood was drawn between 8 and 9 a.m. with syringes moistened with a solution of heparin in saline solution. Samples of the blood in the syringe were taken for determination of histamine and enumeration of eosinophils. The eosinophils were counted according to the method of Randolph (13). The amount of bilirubin in the serum was determined according to the method published by Powell (14).

Free and conjugated histamine in the urine were measured by the method of Roberts and Adam (15). The mean recovery of free histamine by this procedure is 67 per cent (15, 16); no correction has been made for the loss in the computation of results in this paper.

Urine was collected in chemically clean bottles of 2-liter capacity, containing 10 ml. of twice normal hydrochloric acid to maintain the urine at pH 5 or less. Extraction was always started within 24 hours of completing the collection and in the interval the urine was stored in a refrigerator. Tests have shown that no loss of histamine occurs under these conditions.

All values for both blood and urinary histamine reported in this paper are the means of duplicate samples, expressed in terms of the free base. Duplicate determinations were made of the histamine content of 24 different samples of urine and 26 different samples of blood. The duplicate estimates of the free histamine in urine never differed by more than 10 per cent. The differences between duplicate determinations of the conjugated histamine in urine exceeded 10 per cent only twice while the difference between duplicate estimates of the blood histamine was more than 10 per cent on four occasions. In all of the tests, however, there was only one instance in which the difference between duplicates exceeded 20 per cent.

Single determinations of histamine in the blood and urine were made in seven patients with cirrhosis of the liver. All had the typical history and physical findings of advanced portal cirrhosis, and were in a decompensated state with ascites. Blood histamine alone was determined in two patients in the terminal stages of cirrhosis, both being in deep coma at the time.

The effect of cortisone was studied in two of the patients who had high levels of histamine in the blood. After a control period of two days, 50 mg. of cortisone were given every 6 hours for three days, during which daily determinations of histamine were made on the blood and urine. Throughout the investigation, the patients were

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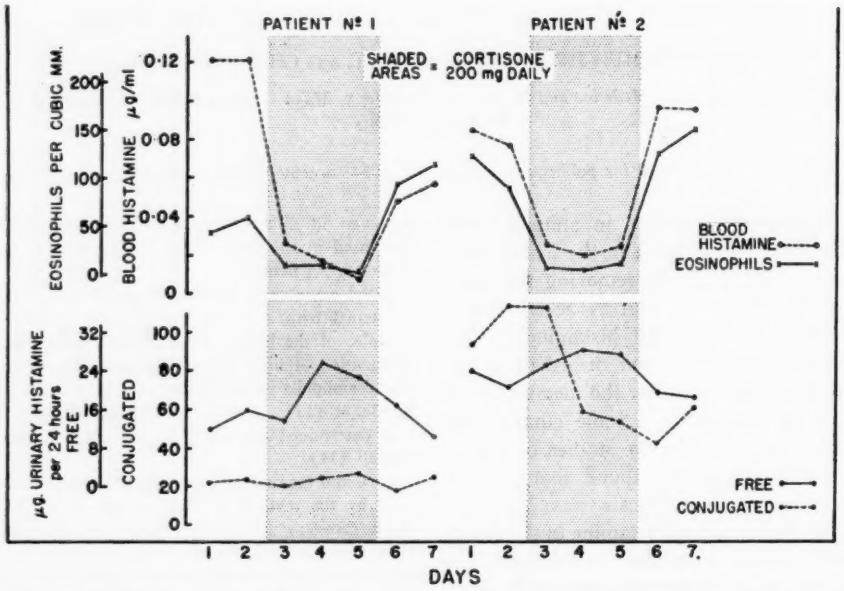

FIG. 1. EFFECT OF CORTISONE ON BLOOD AND URINARY CONCENTRATIONS OF HISTAMINE IN TWO PATIENTS WITH CIRRHOSIS OF THE LIVER

on standard diets which provided almost exactly 100 Gm. of protein per day.

A study was made of 17 patients with proved obstructive jaundice. In most cases the obstruction was due to stricture of the bile passages or to malignant disease. Nine of the patients had a recent history of pruritus but complained of little or no itching when the blood was drawn, and they were placed in a group designated as having mild pruritus. Eight of the patients had moderate to severe pruritus when the blood was taken and they were placed in a group designated as having severe pruritus. In each case, urine was collected during the 24 hours preceding the venipuncture. There was no apparent clinical difference between the two groups, apart from the degree of itching, nor did they differ significantly in the depth of jaundice or in standard measurements of hepatic function.

In one patient with severe pruritus, an assessment was made of the effects of cortisone and of methyl testosterone on the levels of blood and urinary histamine and on the degree of itching.

In order to obtain an estimate, by the methods we used, of the concentration of histamine in the blood of a comparable group of persons in good health, the level of histamine in the blood was determined on at least two different occasions in a group of 12 normal adults.

TABLE I

Histamine content of the blood and urine of seven patients with cirrhosis of the liver

Patient	Blood histamine µg. per ml.	Urinary histamine µg. per 24 hours	
		Free	Conjugated
1	0.121	13.9	28
2	0.081	20.9	104
3	0.037	19.0	100
4	0.067	20.5	147
5	0.051	8.5	227
6	0.040	9.5	54
7	0.075	11.8	109
Mean	0.067	14.9	110

RESULTS

Normal persons. The mean concentration of histamine in the blood of the 12 healthy adults was 0.040 µg. per milliliter with a standard deviation of 0.011 and a standard error of ± 0.002. The maximal and minimal concentrations encountered in the series were 0.067 and 0.012 µg. per milliliter, respectively.

Patients with cirrhosis. The mean concentration of histamine in the blood of the seven patients with cirrhosis (Table I) was 0.067 µg. per milliliter ± 0.011 and this is significantly higher than the mean for the 12 healthy adults ($p < 0.001$).

The values in the patients ranged from 0.037 to 0.121 μg. per milliliter. In contrast, the two patients in the terminal stages of hepatic coma had very low levels of histamine in the blood, 0.017 and 0.010 μg. per milliliter, respectively.

The mean daily excretion of free histamine in the urine of the patients with cirrhosis was 14.9 ± 2.3 μg. per 24 hours, with a range from 8.5 to 20.9 μg. This mean does not differ significantly from that found in another study for normal men (17.7 ± 1.2 μg. per 24 hours) and the range falls within the normal range of 8.0 to 26.9 μg. per 24 hours. The values for conjugated histamine were also within the normal range (Table I).

The administration of cortisone to two of the patients with cirrhosis was accompanied by a decisive fall in the concentration of blood histamine and by a rise in the 24-hour output of free histamine in the urine (Figure 1).

Patients with jaundice. The mean concentration of histamine in the blood of the patients with mild pruritus was 0.052 ± 0.007 μg. per milliliter (Table II) and the mean of the group with severe pruritus was 0.078 ± 0.009 μg. (Table III). The difference between these means is just significant ($p < 0.05$) and both are significantly higher than the mean normal value ($p < 0.02$ and < 0.001, respectively). Only two of the nine values in the group with mild pruritus fell outside the normal range but five of the eight in the group with severe pruritus were at or above the upper limit of the values obtained in the normal group of people (0.067 μg. per milliliter).

The mean values for excretion of free histamine in the urine were 13.5 ± 2.6 and 15.7 ± 3.7 μg. per 24 hours for the mild and severe groups, respectively. These do not differ significantly from one another nor from the mean values for excretion in normal adults.

When cortisone was given to the patient with severe itching, the concentration of blood histamine fell from 0.067 to 0.025 μg. per milliliter, but the itching persisted. The administration of 20 mg. of methyl testosterone per day, resulted in almost complete relief of itching; daily determinations of blood and urinary histamine during the first three days of treatment showed no significant alteration from the pretreatment levels.

TABLE II

Histamine content of the blood and urine of nine patients with obstructive jaundice and mild pruritus

Patient	Serum bilirubin mg. per 100 ml.		Urinary histamine μg. per 24 hours		Blood histamine μg. per ml.
	Direct	Indirect	Free	Conjugated	
1	3.7	1.5	25.5	44	0.050
2	14.0	4.7	23.6	169	0.044
3	6.1	2.1	6.1	23	0.030
4	11.6	3.6	9.1	149	0.045
5	10.1	2.8	6.4	54	0.054
6	5.5	1.5	10.4	24	0.042
7	6.6	1.6	8.5	19	0.032
8	5.2	1.8	10.5	26	0.100
9	13.6	3.5	21.2	35	0.068
Mean	8.5	2.6	13.5	59	0.052

COMMENT

The mean concentration of 0.04 μg. of histamine per milliliter of blood of the 12 normal persons we studied was identical with the mean concentration observed in the blood of 103 medical students by Haworth and Macdonald (17) and identical with the mean concentrations found in the blood of 50 normal persons by Rose and Browne (18) and 20 normal adults by Randolph and Rackemann (19). The values obtained in these series ranged from 0.01 to 0.08 μg. of histamine per milliliter of blood which only slightly exceed the limits noted in our group. Apparently when identical technics are used the concentration of histamine found in the blood of normal persons in the British Isles, Canada, and the United States falls within rather narrow limits. The only sizable series of determinations in which values for normal persons beyond these limits have been obtained is that re-

TABLE III

Histamine content of the blood and urine of eight patients with obstructive jaundice and severe pruritus

Patient	Serum bilirubin mg. per 100 ml.		Urinary histamine μg. per 24 hours		Blood histamine μg. per ml.
	Direct	Indirect	Free	Conjugated	
1	12.9	3.1	39.1	140	0.130
2	1.2	0.3	7.2	20	0.100
3	6.6	3.4	17.6	56	0.057
4	2.0	0.8	14.8	74	0.053
5	13.6	4.2	17.5	6	0.061
6	11.9	2.5	7.9	18	0.083
7	18.0	4.0	14.7	15	0.071
8	17.0	3.7	6.9	153	0.067
Mean	10.4	2.7	15.7	60	0.078

ported by Valentine, Pearce, and Lawrence (20) who obtained a mean value of 0.075 μg. per milliliter with a range of 0.03 to 0.11 in the blood of 17 fasted normal subjects. The upward shift of the mean and of the extreme values suggests some difference in the technics employed.

The decisive decline in the concentration of histamine in the blood and the rise in the output of free histamine in the urine which accompanied the administration of cortisone to the patients with cirrhosis were similar to, though possibly more pronounced than, the changes produced by the hormone in healthy persons (16). This response may reflect a fundamental shift in histamine metabolism which the hormone produces, since it has now been uniformly observed in normal persons, patients with allergic disease and patients with cirrhosis of the liver.

In cirrhosis of the liver and in obstructive jaundice, the mean levels of histamine in the blood were significantly higher than normal. These results confirm those of Chambon and Berthier (6), although the very high values recorded by these authors (up to 0.30 μg. per milliliter) were not obtained.

The finding of an increase in blood histamine accompanied by a normal value for excretion of histamine in the urine of these patients suggests that the high blood levels were not due to liberation of histamine into the plasma, since in such circumstances increased urinary excretion would be expected (21). It is possible, of course, that histamine was retained in the plasma because the kidneys failed to excrete it, but it appears more likely that the high values were due to a disturbance of hepatic function resulting in an increase in histamine in other elements of the blood, possibly in the eosinophilic or basophilic leukocytes. The high values may in some fashion have represented a compensatory mechanism related to the failing liver for when complete dissolution of hepatic function occurred, as was the case in the comatosed patients, the increased quantities of histamine disappeared from the blood.

The results of the study of patients with jaundice and pruritus indicate that there is a relationship between the degree of pruritus and the level of histamine in the blood, in that the more severe the itching the higher the level of blood histamine is likely to be. In cirrhosis, however, high values for blood histamine were found in the absence of itching. Even in the patients with jaundice, the high values for blood histamine did not appear to be the direct cause of the pruritus, since no relief was obtained when the level was lowered by means of cortisone. The elevated blood values simply indicate that the metabolism of histamine is altered when the liver is damaged by cirrhosis or biliary obstruction, and the occurrence of pruritus in the patients with obstruction is apparently related to this altered metabolism.

The relief of itching by the use of methyl testosterone was reported by Lloyd-Thomas and Sherlock in 1952 (22), who commented that the rationale of its use was not known. The lack of effect on the levels of blood and urinary histamine in our patient treated with this hormone suggests that it did not produce the observed relief by correcting the altered metabolism of histamine but rather by some other mechanism.

SUMMARY

The mean concentration of histamine in the blood of patients with cirrhosis and of patients with jaundice caused by biliary obstruction was significantly higher than the concentration in the blood of a group of healthy persons. In the series of patients with jaundice, there was a relationship between the degree of pruritus and the level of histamine in the blood, the more severe the itching the higher the concentration of blood histamine was likely to be.

The values for free and conjugated histamine excreted daily in the urine of the patients with cirrhosis and of those with jaundice were within the limits found in normal persons.

When cortisone was given to two of the patients with cirrhosis, the concentration of histamine in the blood fell decisively and the output of free histamine in the urine increased.

The administration of cortisone to one of the patients who had jaundice with severe pruritus resulted in prompt decline of the concentration of blood histamine without relief of the itching, while administration of methyl testosterone to the same patient resulted in prompt relief of the itching without change in the level of histamine in the blood.

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LIVER AND KIDNEY GLUCOSE-6-PHOSPHATASE ACTIVITY IN CHILDREN WITH NORMAL AND DISEASED ORGANS [1]

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Through the work of the Coris and their coworkers (1–6) over the past twenty years, the structure of glycogen has been clarified and the enzymes necessary for its degradation to glucose have been determined. These enzymes are 1) phosphorylase, 2) amylo-1, 6-glucosidase, 3) phosphoglucomutase, and 4) glucose-6-phosphatase.

In 1949, Marjorie Swanson (7, 8) isolated purified glucose-6-phosphatase from rat liver and found it specific for conversion of glucose-6-phosphate to glucose. She devised a method for the assay of this enzyme which has been used primarily in rats.

Utilizing this technique, a preliminary report of levels of liver glucose-6-phosphatase in 14 patients from 1 month to 10 years of age was made in 1952 (9). A more complete report is herein presented including a total of 37 assays of liver glucose-6-phosphatase on 34 patients and 6 assays of kidney enzyme activity.

METHOD

The liver tissue samples obtained at biopsy varied from 0.043 Gm. to 0.507 Gm. in weight. They were immediately chilled in an iced container after surgical removal. Homogenization and determination of the enzyme activity was usually carried out during the next 2 to 3 hours, while in a few cases the tissue was frozen for later estimation. Those samples obtained at postmortem examination were treated in an identical manner and were obtained at intervals ranging from 1½ to 19 hours after death. Weights of postmortem tissues used for homogenization varied from 0.2 to 0.8 Gm. Specimens were obtained at random from either lobe of the liver. Kidney cortex was obtained at autopsy for renal studies. In four instances, including the case of glycogen heart disease, estimates of heart muscle glucose-6-phosphatase activity showed zero levels.

The liver was ground in a glass homogenizer and diluted approximately 10 fold with 1 per cent glucose solution. Of this, a 0.1 ml. aliquot was incubated with 0.1 ml. of 0.1 molar glucose-6-phosphate and 0.2 ml. of 0.1 M citrate buffer at pH 6.5 at 37° C. for 30 minutes. Simultaneously, an equal sample of buffered liver homogenate was incubated without substrate. The reaction was terminated by immersion of the tubes in cracked ice and by the addition of 1.0 ml. of ice-cold 10 per cent trichloracetic acid. After standing for 5 minutes, a final dilution to 2.5 ml. was made. The mixture was centrifuged for 5 minutes and duplicate 1.0 ml. aliquots were used for inorganic phosphorus determination by a modification of the method of Fiske and SubbaRow (10). The difference in P content of the homogenate incubated with substrate from that incubated alone represented the enzyme activity of the liver. A blank containing all reagents except the liver homogenate revealed no free inorganic phosphorus. The final results of enzyme activity were expressed as mg. P released from substrate per gram of wet liver tissue. Where large amounts of tissue were available, values were expressed in terms of dry weight.

In these cases, since the glycogen occupies so much space, a liver overloaded with glycogen might contain less enzyme activity per gram of weight than a normal liver. To correct this possible error in the comparison of normal livers and livers with excess glycogen content, the nitrogen content of the liver was obtained and the final enzyme activity was expressed as milligram phosphorus per gram of tissue nitrogen.

Repeated estimations of enzyme activity in frozen specimens showed slight loss of activity over several months.

RESULTS

Liver biopsy specimens were obtained from three infants at operation for pyloric stenosis (Table I). These infants were moderately malnourished, had a moderate increase in deposition of fat vacuoles within the liver cells, but were believed to have normal liver function. Necropsy specimens of liver were obtained from six other patients ranging in age from 40 hours to 5½ years. These patients had diseases which did not reveal histological changes in the liver. Specimens were obtained between 3 and 7 hours after death, except in the case of J. C. when the sample was obtained 19 hours after death. The range of values for enzyme activity varied from 3.9 to 5.8 mg. P per Gm. of wet tissue with an average of 4.9 mg. P, or from 165

[1] This work was supported by a grant from Mead Johnson and Company.

TABLE I

Glucose-6-phosphatase activity in normal livers

Patient	Age	Diagnosis	Enzyme activity: mg. P‡ / Gm. wet liver	mg. P / Gm. dry liver	mg. total N§ / Gm. wet liver	mg. P / Gm. N	Microscopic liver findings
J. F.*	6 wks.	Pyloric stenosis	5.0	18.3	20.2	246.	Glycogen—moderate Fat vacuoles—present
K. C.*	4 wks.	Pyloric stenosis	5.4	22.9	18.1	299.	Fat vacuoles—abundant iron present
N. K.*	5 wks.	Pyloric stenosis	5.6	20.1			Fat vacuoles—several
B. A.†	40 hrs.	Premature; Pneumonia	3.9	20.5	23.5	165.	Extra-medullary hematopoiesis
J. C.†	10 days	Duodenal atresia; Kernicterus	5.8	24.8	25.4	229.	Hemosiderosis Hematopoiesis—mild
J. D'A.†	34 days	Thyroid cysts	4.9	16.6	24.3	199.	Fat vacuoles—present
D. W.†	5½ yrs.	Gargoylism; Endocarditis	4.4	19.5	26.0	168.	
J. W.†	5 mos.	Bronchiolitis; Celiac crisis	5.0	23.2	30.3	166.	
M. F.†	5 mos.	Amyotonia congenita	4.5	18.6	19.0	236.	

* Biopsy.
† Autopsy.
‡ P = phosphorus released from substrate, glucose-6-phosphate, by unit measure (Gm.) of liver.
§ N = nitrogen content of liver.

to 299 mg. P per Gm. of liver nitrogen with an average of 214 mg. P (Standard Deviation ± 45 mg.) (Table V). The difference between values obtained at biopsy and necropsy is not significant. Greater variation between results is noted when differences in water content and nitrogen content of the tissues were calculated. This is clearly demonstrated in the case of M. F. whose phosphatase activity of 4.47 mg. P per Gm. of wet tissue is the third lowest figure in the range of normal values, while the enzyme activity of 236 mg. P as expressed per Gm. of tissue N is the third highest value of the group (Table I). Therefore, for standardization of method wherever possible values were expressed in terms of nitrogen content rather than wet weight of tissue.

Glucose-6-phosphatase activity was determined in various diseases and conditions involving the liver (Table II) to evaluate whether enzyme activity is depressed only in specific abnormalities of glycogen storage or whether non-specific insult to the liver will also lower the enzyme activity. In five instances, invasion of the liver by tumor tissue or abnormal cells caused significant reduction of glucose-6-phosphatase activity. In three of seven cases of probable hepatitis or pericholangitis, the specific enzyme activity was more than three standard deviations below the normal, while of the remaining four cases only one was within one standard deviation of the normal. All cases of edema of the liver, passive congestion, biliary and Laënnec's cirrhosis, fatty liver and acute and chronic atrophy were accompanied by reduction of glucose-6-phosphatase activity commensurate with the degree of damage to the liver as observed histologically.

Liver glucose-6-phosphatase activity was studied in two patients with clinical and histological evidence of glycogen storage disease of the liver (von Gierke's disease) (Table III). Liver tissue from one of these patients (W. M.) was examined by Dr. Gerty Cori, who reported that the glycogen was of normal structure (11). Our estimation of liver glucose-6-phosphatase activity in this patient was four and one-half standard deviations below the mean. This patient also had an associated glycogen-containing liver tumor which had less enzyme activity per gram of tissue than did the more normal areas of the liver.

T. A. had a typical clinical picture of glycogen storage disease with hypoglycemia, ketosis and lactic acid acidosis and a liver which extended into the pelvis. The liver at biopsy contained large amounts of glycogen which did not disappear after

24 hours; its enzyme activity was only slightly greater than that of W. M. Death occurred at age 3 years 7 months. Autopsy was not permitted.

Postmortem studies of glycogen content and enzyme activity of tissue were performed in a 2-month-old infant (D. B.) who was observed from

TABLE II

Liver glucose-6-phosphatase activity in miscellaneous diseases of the liver

Patient	Age	Diagnosis	Enzyme activity: mg. P‡ / Gm. wet liver	mg. P / Gm. dry liver	mg. total N§ / Gm. wet tissue	mg. P / Gm. N	Liver pathology
M. C.†	14 mos.	Neuroblastoma					
		a) normal liver	3.4	14.8	23.0	147.	Edema of liver
		b) tumor liver	0.6	3.3	21.8	26.	
S. B.†	4½ yrs.	Nephrosis; Chronic glomerulo-nephritis	2.7	12.3	23.2	117.	Edema of liver
J. P.†	19 mos.	Letterer-Siwe disease	2.4	10.4	28.4	84.	Reticulo-endotheliosis; Giant cells; Kupffer cells increased
M. W.†	2 yrs.	Reticulo-endotheliosis	0.8	3.3	29.0	26.	Reticulo-endotheliosis
J. R.†	1½ yrs.	Leukemia	2.5	11.1	34.6	103.	Leukemic infiltration; Multinucleate liver cells
G. H.*	9 yrs.	Rhabdomyo-sarcoma	3.7	14.9	23.4	158.	Some portal fibrous tissue and bile duct proliferation
S. D.*	3 mos.	Hepatitis	1.6	6.4	20.9	76.	Hepatitis;
†	10 mos.	Pneumonia (?)	3.2	12.3	33.0	97.	Liver healing
A. S.†	30 days	Cirrhosis; Kidney abscesses	1.7	8.6	20.0	85.	Cirrhosis
R. T.†	14 mos.	Infectious mono-nucleosis	0.9	3.1	17.9	43.	Acute hepatitis; Subacute yellow atrophy; Fatty
T. S.*	3½ yrs.	Subacute yellow atrophy	1.0				Subacute yellow atrophy with localized regeneration
C. S.†	5 wks.	Chronic intestinal obstruction	6.4	29.8	27.3	234.	Mild pericholangitis
C. So.*	2½ yrs.	Cirrhosis	3.6	14.3	30.0	146.	Pericholangitis; Periportal cirrhosis
E. B.*	4 mos.	Hepatitis	0.08				Multinucleated liver cells; Cirrhosis
R. J.*	2½ mos.	Bile duct atresia	3.7		25.6	146.	Pericholangitis; Biliary cirrhosis
G. D.†	13½ yrs.	Fibrocystic disease of pancreas	2.1	10.7	22.1	96.	Biliary cirrhosis focal; Cysts of intrahepatic bile ducts
R. M.†	3 yrs.	Lead poison (?)	0.2	7.2	25.8	6.	Laennec's cirrhosis
C. W.†	3 yrs.	Low gamma globulin; Intestinal obstruction	2.4	9.6	22.8	104.	Fatty; Binucleate liver cells
L. A.†	4½ mos.	Congestion of liver; Nephro-calcinosis	2.7	10.4	27.6	97.	Fatty
A. L.*	4½ yrs.	Congestion of liver; Tumor (?)	3.4	13.6	28.5	119.	Acute passive congestion; Central atrophy
Ja. Co.*	12 mos.	Cysts of liver; Hypoglycemia	3.6	14.6	24.6	146.	Some portal areas large, some small; Anomalous bile ducts
G. T.†	1½ yrs.	Spastic diplegia; Pneumonia; Cachexia	1.9	7.9	31.3	60.9	Atrophy of liver
G. M.*	4 mos.	Hepatitis	2.7	10.5	29.9	90.	Hepatitis; Fatty; Portal cirrhosis mild

* Biopsy.
† Autopsy.
‡ P = phosphorus released from substrate, glucose-6-phosphate, by unit measure (Gm.) of liver.
§ N = nitrogen content of liver.

TABLE III

Liver glucose-6-phosphatase activity in glycogen storage disease

Patient	Age	Diagnosis	Enzyme activity: mg. P‡ / Gm. wet liver	mg. P / Gm. dry liver	mg. total N§ / Gm. wet tissue	mg. P / Gm. N
W. M.†	10 yrs.	a) Liver storage	0.23	0.80	23.39	9.7
		b) Liver tumor	0.16	0.48		
T. A.*	5½ mos.	Liver storage	0.46	1.35		
D. B.†	2 mos.	Heart storage	6.86	22.46	20.07	342.0

* Biopsy.
† Autopsy.
‡ P = phosphorus released from substrate, glucose-6-phosphate, by unit measure (Gm.) of liver.
§ N = nitrogen content of liver.

birth with suspected glycogen storage disease of the heart. Clinically there was cardiomegaly with progressive T wave changes in the electrocardiogram, a large tongue and a liver which measured 5 cm. below the costal margin, fasting blood sugar levels of 60, 80, 89, 84 and normal glucose, fructose and galactose tolerance tests. Large deposits of glycogen were present in heart and skeletal muscle. Dr. Cori studied the glycogen in these tissues and reported its structure to be made up of glucose radicals in normal chain length. Using a slightly different method (12), Dr. Cori reported that the liver glucose-6-phosphatase was normal (11). Our estimations of glucose-6-phosphatase activity of the liver measured 342 Gm. P released from substrate per gram of liver nitrogen. This is 2.9 standard deviations above our normal average for liver enzyme activity.

Postmortem analysis of kidney cortex for enzyme activity (Table IV) showed much lower values in the two normal kidneys (av. = 119 mg. P per Gm. N) than was obtained in the livers. Patients with renal disease or infiltration had enzyme activity much lower than the normal, while the kidney cortex of one patient (W. M.) with glycogen storage disease of the liver had only five per cent of the expected normal activity. Enzyme activity of the kidney cortex of D. B., the patient with glycogen storage disease of the heart, was not tested by us but was determined by Dr. Cori (11).

COMMENT

Within the limits of the small samples available, these studies show the relative constancy of glucose-6-phosphatase activity of tissue of normal

TABLE IV

Glucose-6-phosphatase activity of kidney cortex in normal and pathological conditions (postmortem specimens)

Patient	Age	Diagnosis	Enzyme activity: mg. P* / Gm. wet kidney	mg. P / Gm. dry kidney	mg. total N† / Gm. wet tissue	mg. P / Gm. N
M. F.	5 mos.	Pneumonia; Amyotonia congenita	2.6	14.8	22.2	115.
J. W.	5 mos.	Celiac crisis	2.3	15.1	18.7	122.
A. S.	30 days	Liver cirrhosis; Kidney abscesses	1.7	8.6	20.0	85.
L. A.	4½ mos.	Nephro calcinosis; Liver congestion	1.3	6.7	21.0	61.
M. W.	2 yrs.	Reticulo-endotheliosis	0.6	3.0	26.1	22.
W. M.	10 yrs.	Glycogen liver disease	0.18	0.8		

* P = phosphorus released from substrate, glucose-6-phosphate, by unit measure (Gm.) of kidney.
† N = nitrogen content of kidney.

TABLE V

Liver glucose-6-phosphatase activity

	mg. P / Gm. wet liver		mg. P / Gm. dry liver		mg. N / Gm. wet liver		mg. P / Gm. N	
	M.*	S.D.†	M.	S.D.	M.	S.D.	M.	S.D.
Normal livers	4.9 (9)‡	0.6	21.0 (9)	3.0	23.0 (8)	4.0	214.0 (8)	45.
All liver disease except glycogen storage of liver	2.5 (23)	1.4	11.0 (20)	5.0	26.1 (21)	4.0	104.0 (21)	49.
Liver in 2 cases glycogen storage	0.4 (2)		1.1 (2)		23.0 (1)		9.7 (1)	
Liver in 1 case glycogen storage of heart	6.9		22.5		20.1		342.0	

* Mean.
† Standard deviation.
‡ ( ) = Number of examinations.

infants and children and indicate the degree of suppression of this enzyme's activity in various diseases. The only circumstances in which its activity seems to approach zero are in those cases of abnormal storage of glycogen in liver and kidney. Autolysis studies in these instances have also shown very slow breakdown of glycogen structure.

Of interest is the one case of glycogen storage disease of the heart in which liver (and kidney) glucose-6-phosphatase activity was greater than normal. There also appeared to be greater storage of liver glycogen than was expected, and the structure of the glycogen was found on analysis to be normal. This infant also had relatively high fasting blood sugar levels for his age. One wonders whether these findings usually accompany a defect in muscle glycogen breakdown or whether this is unique.

SUMMARY

Studies of glucose-6-phosphatase activity of liver and kidney tissue, using the method of Swanson, were performed on biopsy and autopsy material obtained from patients varying in age from 40 hours to 13½ years. The normal range of activity was determined and compared with the lower values obtained in various diseases affecting liver and kidney. Enzyme activity in two cases of glycogen storage disease of the liver was almost absent, while the liver enzyme activity in one case of glycogen storage disease of the heart was abnormally high.

Addendum: Liver glucose-6-phosphatase activity in a biopsy specimen from a three-month-old infant with ideopathic hypoglycemia, not due to von Gierke's disease or pancreatic adenoma, was 6.2 mg. P per Gm. of wet tissue, or 252 mg. P per Gm. N.

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THE PATHOGENESIS OF CYSTINURIA. I. CHROMATOGRAPHIC AND MICROBIOLOGICAL STUDIES OF THE METABOLISM OF SULPHUR-CONTAINING AMINO-ACIDS

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Recent studies of cystinuria from this laboratory (1) have suggested that the disease does not, as had been assumed by most previous workers, involve an innate inability to metabolize one or other of the sulphur-containing amino-acids. On the contrary the defect responsible for the life-long constant excretion of large amounts of cystine appears to be due to a peculiarity only of renal function, that is, to a "low renal threshold" for cystine presumably due to diminished tubular reabsorption. This view was based in the first instance on the finding of large quantities of lysine, arginine, and perhaps ornithine, in the urine, a fact not readily explainable by Garrod's simple concept of an "inborn error of metabolism" of cystine, but quite consistent with the findings in certain other renal amino-acidurias. It was further supported by preliminary chromatographic and microbiological determinations of plasma cystine levels, since they were found to be no higher in cystinuric subjects than in normal subjects. This has since been confirmed by polarographic determinations (2). The renal theory had already been suggested by other workers but had only been subjected to one earlier incomplete investigation, which appears to have been largely overlooked (3).

Although the renal theory explains readily all of the spontaneously occurring clinical and biochemical phenomena it is still difficult to fit into it much of the work of others in which the metabolism of sulphur-containing amino-acids had been investigated (4). This work is often contradictory and suffers greatly from the inadequacy of the methods of analysis then available. The weight of evidence however indicated that cystine given by mouth to a cystinuric subject did not result in an increased excretion of cystine. Methionine and cysteine on the other hand did increase the cystine output. This had been interpreted as an error of metabolism not of cystine, but of cysteine, methionine acting only as a source of cysteine (4).

In the present paper we report fuller investigations on the changes in plasma and urine amino-acids in two normal patients and two cystinuric patients after feeding loading doses of cystine, cysteine, and methionine. Only acute experiments on fasting subjects were carried out as this technique should exaggerate the metabolic changes more than the experiments done by others on the basis of 24-hour urine collections. Paper chromatography was chosen as the chief method of analysis owing primarily to its high specificity but also because of its ability to indicate changes in concentration of every amino-acid present in appreciable quantity, the importance of doing which having already been stressed (5). Owing to the only semi-quantitative nature, however, of paper chromatography, microbiological assays for cystine were also carried out in most of the experiments. As this quantitative method is also subject to question, especially in the case of cystine, the present report was held up until a more extensive check had been carried out by polarography (6).

METHODS USED AND CASES STUDIED

Cases. The experiments were performed on two normal subjects (G. J. and D. F.) and on two cystinuric subjects (L. L. and J. H.). G. J. was a male age 33, weight 81.8 Kg., D. F., a female age 23, weight 52.3 Kg. The cystinuric subjects (under the same initials) are described in a little more detail by Dent and Rose (1). Briefly, L. L. was a taxi driver aged 41 (weight 73 Kg.) who had had attacks of renal colic since 17 years of age, and had occasionally passed small smooth greyish white stones proved on analysis to be of cystine. X-ray showed a small opacity to be present in the left renal area. J. H. was a fitter aged 23 (weight 68 Kg.) who had had three attacks of renal colic at 11, 19, and 22 years of age. There

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was no X-ray evidence of stones. His urine usually deposited typical cystine crystals on standing. The urine of both cystinuric subjects gave the typical cystine-lysine-arginine pattern on chromatographic analysis.

Procedure during experiment. The subjects were fed separately each of the three amino-acids, at least two days intervening between each test. The quantities taken were equivalent in respect of their sulphur content, *i.e.*, 5 g. of L-cystine, 6.2 g. of DL-methionine and 6.5 g. of L-cysteine hydrochloride. The latter was neutralized with NaOH to pH 7.0 immediately before ingestion. The subjects ate nothing from the previous evening and until the experiment was over. They were kept lying on a couch throughout the test in order to minimize the changes in glomerular filtration rate known to occur with changes in posture and activity. At the beginning of test (0 hour) the amino-acid was drunk either dissolved or suspended in water, and a liberal water intake was maintained subsequently to ensure adequate urine flow and bladder emptying. Blood samples were obtained immediately before the amino-acid was taken and again at ½, 1, 2, 4, and 6 hour intervals. The blood was taken into a heparinized syringe, immediately centrifuged and the plasma separated and stored at 4°. Specimens were rejected if any trace of hemolysis was present. The urine specimens were collected at 0, 1, 3, 5, and 7 hours. The last time of emptying the bladder before the experiment was also noted so that the specimen collected at 0 hour could also be timed. All the urines were preserved with thymol at 4°.

The normal subject, D. F., felt nauseated shortly after swallowing the cysteine and vomited shortly before the 1 hour blood sample was taken.

Chromatographic analyses. The plasma samples were prepared for analysis by, first, ultrafiltration to remove the proteins, the method used being that described by Dent and Schilling (7), secondly, by electrolytic desalting, using a micromodification (8) of the ingenious apparatus devised by Consden, Gordon, and Martin (9). The urine samples were run without any pretreatment, the volume taken for each analysis being that passed in a given time interval, usually 2 sec. or 2/5 sec.

Two-way paper chromatograms (18 by 22 inches) were used exclusively for the amino-acid analyses. Phenol/NH_3 and collidine-lutidine/Et_2NH were the two solvents used (10). Each sample of desalted plasma ultra-filtrate (625 µl.) was run alone and again after oxidation with 10 µl. H_2O_2 (30 per cent) and 10 µl. 0.1 per cent ammonium molybdate so as to enable methionine and cystine (and/or cysteine) to be detected in the form of methionine sulphone and cysteic acid, respectively (11).

Each set of 5 to 6 samples of urine or of desalted ultrafiltered plasma from one experiment was run simultaneously in the chromatogram cabinet. The resulting chromatograms were strictly comparable with each other and on being laid out side by side a most accurate picture of the changes taking place in plasma amino-acid levels and urine outputs could be obtained by merely comparing in turn the strengths and sizes of each amino-acid spot as it appeared in successive chromatograms. In order to determine approximately the plasma cystine (and/or cysteine) concentration a known but varying quantity of cysteic acid was placed on each chromatogram about 4 cm. from the site of the plasma or urine sample. The quantities chosen covered the full range of cystine levels anticipated in the samples and were as far as possible placed on chromatograms likely to contain similar amounts in the sample. In this way each cysteic acid spot obtained from the oxidized cystine in the sample could be matched by eye with a similar strength spot on the same chromatogram or on one of the others in the same batch, and a semi-quantitative estimate obtained. This method was considered to be very accurate when used for determining relative strengths of an amino-acid in a series of samples from one experiment, and we were impressed by the definiteness of the serial changes shown as varying strengths of the spot on the chromatograms in, say, the cystine levels of plasma after feeding cysteine (see later). However, a fairly constant loss of cystine always occurs during the H_2O_2 oxidation so we expected the absolute values to be on the low side.

As a further check, each urine sample was tested directly under standard conditions, for cystine and for cysteine by the cyanide/nitro-prusside test (12).

Microbiological assays. Plasma samples were first prepared for assay as previously described for chromatographic analysis, except that the electrolytic desalting was not carried out since preliminary experiments showed that the small amounts of mercury introduced into the samples proved to be toxic to the assay organism. After ultrafiltration, each plasma sample was diluted, where necessary, with distilled water to the appropriate volume before assay for cystine at 2 to 4 levels in duplicate whereever possible.

The cystine assays were carried out using Leuconostoc mesenteroides P-60 since, according to Riesen, Spengler, Robblee, Hankes, and Elvehjem (13), this organism is the most specific in its response to cystine as distinct from its simple derivatives, and the standard curves are more reproducible than those obtained with any other strain of bacteria. The most suitable medium was found to be an oxidized peptone medium which was essentially that described by Lyman, Moseley, Wood, and Hale (14) with minor modifications chiefly in respect of the mineral and vitamin concentrations.

Urine samples were diluted from 1:25 to 1:500 (by vol.) depending on the concentration of cystine in the urine. In some samples containing very high concentrations, crystallization of the cystine had taken place before they were received for assay, and in such cases, careful homogenization was necessary to ensure reproducible results. The diluted urine samples were assayed in duplicate at 3 to 5 levels.

The microbiological assay of the true free cystine in urine normally presents considerable difficulty owing to the presence of many diverse substances stimulating to the growth of the organism. The problem was overcome in the present experiments in the following manner: A solution was made by dissolving Difco peptone (50 g.) in

TABLE I

Cystine in plasma and urine after 5.0 g. of l-cystine (microbiological assay)

	Time (*hr.*)	−½	0	½	1	2	3	4	5	6	7
D. F. (normal)	Plasma (*μg./ml.*)		11	12	—	9		—		—	
	Urine (*μg./min.*)		← 37 ←	31 →		← 58 →		← 48 →		← 44 →	
J. H. (cystinuric)	Plasma (*μg./ml.*)		11	13	12	8		10		15	
	Urine (*μg./min.*)		← 585 ←	838 →		← 818 →		← 630 →		← 788 →	
L. L. (cystinuric)	Plasma (*μg./ml.*)		9	—	10	11		11		—	
	Urine (*μg./min.*)		← 558 ←	558 →		← 588 →		← 653 →		← 465 →	

a mixture of urine (90 ml.), water (360 ml.) and concentrated HCl (50 ml.). To this solution, hydrogen peroxide (15 ml. of 100 vols.) was added and the whole was allowed to stand overnight. The mixture was then treated in the usual manner as described by Lyman, Moseley, Wood, and Hale (14) and diluted to one liter. The requisite salts, essential vitamins, methionine, tyrosine, and tryptophan were added to 400 ml. of this solution to make one liter, of double strength medium. The assay was carried out in the same manner and using the same organism as for the plasma assays. The sensitivity of the organism to cystine was increased considerably in this unusual medium and the assay was much improved. In addition, wherever possible, in the making up of the peptone-urine medium, the urine from the particular subject under investigation, whether control subject or cystinuric subject, was used. For this purpose, a separate, bulked, 24-hour sample of urine was collected before the feeding experiments were carried out.

Despite the precautions taken, the assays of both plasma and urine samples still left something to be desired, partly no doubt due to the presence of peptides in the former samples and partly due to the peculiar assay difficulties in the case of cystine referred to previously by one of us (15). Furthermore, when the present work was carried out, the improved procedure of Camien and Dunn (16) had not been published and it would seem likely, therefore, in view of these authors' results, that the present microbiological assay results for both plasma and urine are relatively high. Nevertheless the results were reproducible and the general trends reliable.

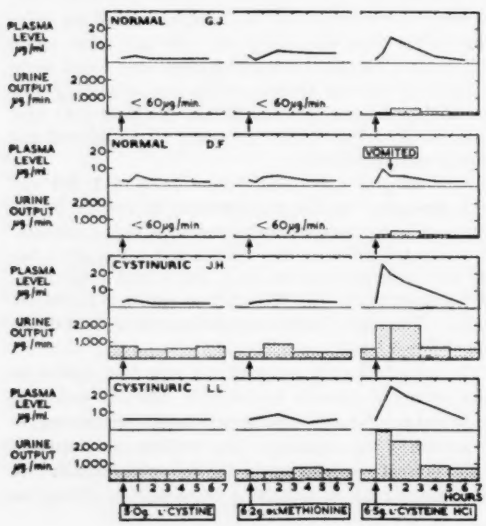

FIG. 1.

RESULTS

Chromatographic assays

The relative changes in plasma level and urinary output of cystine following the ingestion of the three amino-acids can be seen from the graphs in Figure 1.

These changes and those in plasma levels of other amino-acids were as follows:

(a) In no subject was the plasma cystine appreciably raised after giving the cystine. Following its ingestion there was a slight increase in α-amino-n-butyric acid level in the earlier samples from the normal subject, D. F. The cystinuric subject, J. H., showed a slightly higher than normal concentration of α-amino-n-butyric acid in all specimens obtained in the fasting state and following the ingestion of cystine there was a further slight increase in α-amino-n-butyric acid and methionine which was maximal at 1 hour. These changes were not reflected in the urine outputs.

(b) Following the ingestion of methionine the plasma cystine level showed no consistent changes, but the methionine rose to a high level maximal at 1 to 2 hours, and remained still appreciably raised after 6 hours. The normal subjects and cystinuric subjects behaved similarly and in all cases there was also a definite slight rise in α-amino-n-butyric acid level which was usually maximal in the 6-hour specimen. The urine output of methionine was greatly increased in all specimens passed while the plasma level was raised.

(c) Following the ingestion of cysteine there were in all cases large rises in plasma cystine levels.

TABLE II

Cystine in plasma and urine after 6.2 g. of dl-methionine (microbiological assay)

	Time (*hr.*)	−½	0	½	1	2	3	4	5	6	7
D. F. (normal)	Plasma (*μg./ml.*)		10	9	6	5		10		6	
	Urine (*μg./min.*)		← 30	← 70 →		← 60 →		← 90 →		← 50 →	
J. H. (cystinuric)	Plasma (*μg./ml.*)		11	17	20	19		21		17	
	Urine (*μg./min.*)		← 426	← 683 →		← 1740 →		← 715 →		← 703 →	
L. L. (cystinuric)	Plasma (*μg./ml.*)		17	—	14	12		13		—	
	Urine (*μg./min.*)		← 564	← 509 →		← 700 →		← 756 →		← 646 →	

The only other change of note was in the cystinuric subject, J. H., who (as after taking cystine) showed a slight rise in α-amino-n-butyric acid level.

An important negative finding was the absence of any detectable changes, in any of the experiments, in the plasma level or urinary output of lysine and of arginine.

Twenty-four hour samples of urine of the cystinuric subjects J. H. and L. L. were collected while they were on the ordinary hospital diet. Chromatographic analysis of the cystine output gave in both cases figures of the order of 800 to 900 mg. per 24 hour.

The cyanide/nitro-prusside testing of all the urines gave colour reactions for the cystine which corresponded approximately to the concentrations expected on the basis of the chromatographic assay. The nitro-prusside test also showed that traces of cysteine were present in some of the urines passed at the time when the maximal additional cystinuria was taking place after ingesting cysteine. At other times the urinary amino-acid appeared to be entirely cystine and not cysteine.

Microbiological assays

The results are shown in Figure 2 and Tables I–III. These confirm the main chromatographic findings as regards the changes in plasma cystine level and urine output (see above).

DISCUSSION

After ingesting a large dose (5 g.) of cystine, only slight changes in plasma cystine level occurred. This confirms Hier (17), who used microbiological assays, and Brown and Lewis (3), who used a chemical method. We also confirmed that no increased cystine output occurred in either normal or cystinuric subjects. After methionine, the results were more ambiguous. The plasma cystine levels probably rose slightly and this was definitely followed in the cystinuric patients by an increased cystine output although the change in the normals, if any indeed occurred, was too small to be detectable. Spectacular changes however occurred after feeding cysteine. In all four subjects the plasma cystine level rose considerably and was associated with an increased urinary output. We appear, therefore, to have a ready explanation of the results of the metabolic experiments done by earlier workers, namely, the cystine output in the urine in any given subject is dependent mainly, at least, on the plasma cystine level, and the extent of cystine output at a given plasma level depends on whether the patient is cystinuric or otherwise, as the cystinuric patients always leak out into their

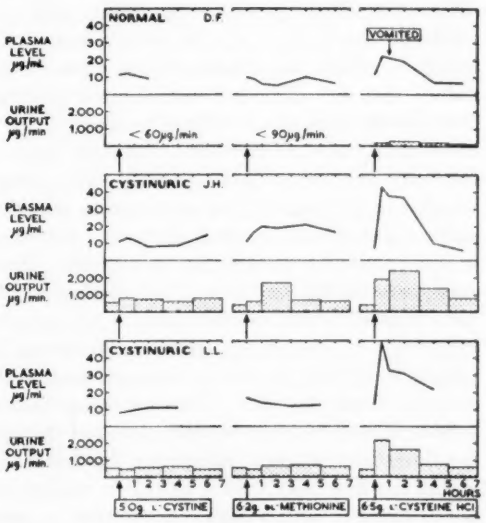

FIG. 2.

TABLE III

Cystine in plasma and urine after 6.5 g. of l-cysteine HCl (microbiological assay)

	Time (*hr.*)	−½	0	½	1	2	3	4	5	6	7
D. F. (normal)	Plasma (*μg./ml.*)		11	22	—	19		7		7	
	Urine (*μg./min.*)		← 20 ←	140 →		← 230 →		← 140 →		← 60 →	
J. H. (cystinuric)	Plasma (*μg./ml.*)		6	43	38	37		10		6	
	Urine (*μg./min.*)		← 463 ←	1905 →		← 2489 →		← 1465 →		← 857 →	
L. L. (cystinuric)	Plasma (*μg./ml.*)		13	48	33	31		22		—	
	Urine (*μg./min.*)		← 479 ←	2158 →		← 1697 →		← 780 →		← 612 →	

urine more cystine than the normal subjects at all plasma cystine levels. This finding supports the "low renal threshold" theory of Dent and Rose (1) and is strong evidence against the "error of metabolism" theory of Garrod (18) and others. The equally rapid fall in the high plasma cystine levels shown by both normal and cystinuric subjects after taking cysteine is an important piece of evidence against the latter theory. If a true "error of metabolism" were present a "diabetic" type of cystine tolerance curve would have been expected in the cystinuric subjects.

Our results however still leave some puzzling features inadequately explained. Why does the plasma cystine level rise so much more after giving cysteine than after cystine? Brand, Cahill, and Harris (4) sought to explain the results of their metabolic experiments (in which the urine only was examined) by postulating that there were different metabolic pathways for cystine and cysteine and that it was only the metabolism of the cysteine (which was chiefly derived from methionine) that was disturbed in cystinuric subjects. We find this suggestion difficult to accept in view of the ready interconvertibility that must exist in the body between —S—S— and —SH groups whether in the form of free amino-acids, peptides, such as glutathione, or whole proteins. Moreover in our results we have found no essential differences between the way normal and cystinuric subjects metabolize these amino-acids. Our tentative hypothesis is, therefore, that the differences in plasma levels may be due to different rates of absorption from the gut. Cysteine being readily soluble in water is very rapidly absorbed through the intestinal mucous membrane, probably all within the first hour, and it therefore reaches the portal blood in such a high concentration as temporarily to saturate the mechanisms for maintaining a constant blood level of cystine. The large dose of cystine on the other hand remaining almost entirely undissolved in the intestinal fluids is only taken into the portal blood relatively slowly and so is adequately dealt with by the liver and never reaches the systemic blood in appreciably raised concentrations. Another possibility is that the cystine is partly decomposed, say, into H_2S and alanine, by bacteria while in the gut, so preventing some from being absorbed at all. Our experiments with cystine were sufficiently prolonged for maximal absorption since earlier work on the increased urinary sulphur output in the urine after giving cystine by mouth has shown that the cystine must have been largely absorbed in some form or other in the 6-hour space of our experiment.

It has been repeatedly found in rats (Dent and Harbury, Unpublished work) that a similar cystinuria can be induced by feeding a single large dose of cysteine but not by the same dose of cystine. So, whatever the explanation, we are convinced that this is a general phenomenon which is not confined to cystinuric subjects. We can only find one reference in the literature to the feeding of cysteine to normal humans namely, Hess and Sullivan (19) who gave 2.6 g. of the hydrochloride but reported no increased output of cystine in the 24-hour urine. Our present results do not confirm this and provide evidence that there is probably no clear-cut difference between the metabolism of cysteine by normal and cystinuric subjects. Increased cystine output after cysteine feeding cannot therefore be used as a diagnostic test for cystinuria (Freudenberg [20]).

SUMMARY

1. Chromatographic analyses of plasma and urine have been carried out during the 6 hours following the ingestion of large single doses of cys-

tine, cysteine, and methionine by two normal and two cystinuric subjects. Most of the samples were also assayed by microbiological methods.

2. The changes observed showed similar trends in both normal and cystinuric subjects.

3. Following ingestion of cystine only slight, possibly insignificant, changes in plasma cystine level were detected. There was no change in urinary cystine output.

4. Following ingestion of methionine there were only slight rises in plasma cystine levels but a slight increase in urinary cystine output occurred in the two cystinuric subjects. The plasma contained very high concentrations of methionine.

5. Following ingestion of cysteine there were very large rises in plasma cystine levels. Greatly increased urinary cystine outputs were also observed in both normal and cystinuric subjects.

6. The changes in output of cystine in the urine in any given subject roughly followed changes in plasma cystine level. In the two cystinuric patients, however, the urinary output was greatly increased over normal at all plasma levels.

7. These results are taken to support the suggestion of Dent and Rose (1) that in cystinuria the body tissues as a whole show no "error of metabolism" for cystine in the sense in which this term was used by Garrod. The biochemical findings in cystinuria can be better explained on the basis only of kidney tubule dysfunction.

8. Consistent slight changes occurred in the levels of α-amino-n-butyric acid in some of these experiments, as if to suggest that it is closely related metabolically to the sulphur containing amino-acids (see also Dent and Schilling [7]).

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THE PATHOGENESIS OF CYSTINURIA. II. POLAROGRAPHIC STUDIES OF THE METABOLISM OF SULPHUR-CONTAINING AMINO-ACIDS

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The preceding paper (1) in this series describes some paper chromatographic and microbiological analyses of plasma and urine during acute experiments in which loading doses of cystine, cysteine, and methionine were given by mouth to normal and cystinuric subjects. Cysteine alone caused large rises in plasma cystine levels and urine outputs in both normal and cystinuric subjects. In all cases, urine output varied directly with plasma cystine levels, but in the cystinuric subjects the urine output was very much greater for any given plasma level than in the normal subject. The results were taken as strong evidence in favour of the renal theory of cystinuria, namely that the high cystine output of a cystinuric patient is due not to an innate inability to metabolize cystine or other sulphur-containing amino-acids but to a "low renal threshold" for cystine, presumably from defective tubular reabsorption. The methods of analysis used though highly specific qualitatively were not, however, considered sufficiently accurate quantitatively to enable renal clearances to be calculated.

In this paper we have repeated in greater numbers experiments of the type described in the preceding paper (1) using an almost identical procedure for timing and collecting samples of plasma and urine. The cystine determinations, however, were done exclusively by polarography. This method, depending upon entirely different principles, is likely to be specific in the circumstances. There is no convincing evidence for the presence in these fluids (after deproteinization when necessary) of appreciable quantities of other substances which might interfere with the analysis owing to their containing disulphide (—S—S—) or thiol (—SH) groups.

In addition we have been able to study during these experiments the cystine clearances of normal and cystinuric subjects and in one case also of an "incomplete cystinuric" subject. A discordant series of cystine feeding experiments was shown to be due to the inadvertent use of partially racemized cystine, an important possibility of error which appears to have been overlooked by earlier workers. This led to a further study of the metabolism of DL-cystine. We have also investigated in a preliminary manner the possibility that there exists at some point in the kidney tubule a common mechanism for the reabsorption of cystine, lysine, arginine, and ornithine.

MATERIALS AND METHODS

Twenty-one observations were made on nine normal adult subjects, 10 observations on six cystinuric adults and one cystinuric child and two observations on one "incomplete cystinuric" adult (see later). Relevant clinical details of the cystinuric subjects are given in Table I. In all other respects they also conformed to the definition of the disease that Dent and Rose (2) have proposed. S. L. and A. L. were identical twins. Further details of cases J. H., S. L., A. L., and G. S. (under the same initials) may be found in the paper by the latter (2). The subject described as having "incomplete" cystinuria was healthy and symptom free; he excreted between 120 and 180 mg. per 24 hr. of cystine (2 to 3 times normal). He was believed to be one of the heterozygous forms of the condition (3).

The amino-acids fed (dissolved in or washed down with water) in the various experiments were: L-cystine (5.0 g.): L-cysteine hydrochloride (6.0 g.): DL-methionine (6.25 g.): DL-cystine[3] (5.0 g.)—this was prepared by the method of Hoffman and Gortner (4); a mixture of L-arginine hydrochloride, L-lysine hydrochloride and DL-ornithine hydrochloride (5.0 g. of each); glycine (15.0 g.). The hydrochlorides were neutralized with $NaHCO_3$ immediately before feeding.

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[3] We use the term DL-cystine with the knowledge that it is impossible at the moment to go further into the matter of differences in behaviour between racemic and meso-cystine.

The procedure in each experiment and the schedule of specimen collections was exactly the same as that described in the preceding paper in this journal (1), namely blood at 0, 0.5, 1, 2, 4, and 6 hr. intervals after feeding amino-acid; urine at 0, 1, 3, 5, and 7 hr. intervals. The subjects however were not all maintained recumbent. The rate of urine production was about 1 to 2 ml. per min. (It was fortunate that larger diureses were not attempted, as we have some recent results [unpublished] which indicate that the cystine "clearances" rise considerably with a high diuresis in a patient with stones as if dissolution of the stones was occurring. This could have produced considerable artefacts in acute experiments of the type done here.)

In addition to the amino-acid experiments, control studies following the same schedule but without amino-acid ingestion were carried out in three normal and one cystinuric subject.

In one cystinuric patient simultaneous inulin (5) and endogenous cystine clearances were carried out. Short collection periods were used with a marked diuresis. A cystinuric subject (J. H.) with no radiological evidence of stone formation was therefore chosen for this experiment.

The cystine was determined by polarography (6, 7) using a Tinsley ink recorder. In each urine analysis 1 ml. of the diluted urine was added to 3.9 ml. of buffer made up from equal volumes of 0.125 N ammonia and 0.125 M ammonium chloride and containing 4.0 g. per 100 ml. of sodium sulphite to avoid interference by oxygen. Immediately before the analysis, 0.1 ml. of M/10 cobaltous chloride was added. The plasma (0.5 ml.) was first dialyzed against distilled water (2.5 ml.) for 3.5 hr. at 4° C. in the type of apparatus described by Hamilton and Archibald (8). One ml. of the dialysate was analyzed as in the urine method. All determinations were done in duplicate. Our standard curve gave a straight line relationship between wave height and quantity of cystine in the cell between the range of 1 to 10 μg. of cystine. Recovery of cystine added to plasma or urine was ± 10 per cent. The polarograph, like most other methods of analysis, determines cystine and cysteine together, but it is most unlikely that cysteine exists as such in either plasma or urine. In plasma the high oxygen tension and pH would favour its rapid oxidation to cystine. Moreover a direct chemical method of determination has shown that no cysteine can be detected in normal plasma (9). This still leaves open the possibility that some cysteine is present during the peak rise of blood level after feeding cysteine (see later). As indicated in the preceding paper (1) and confirmed again here the urines at these periods give only very weak direct cyanide nitro-prusside reactions for cysteine, so cysteine was probably also absent from the blood. We have assumed here that the polarographic method is specific for cystine/cysteine. We note however that the recent method of Moore and Stein (10) for amino-acid assay while agreeing closely with the polarographic cystine determinations in plasma, and in cystinuric urines (11), gives consistently lower results with normal urine (12). We have confirmed the discrepancy in this laboratory using both methods of analysis on the same specimens of urine (D. F. Evered—unpublished). The matter remains *sub judice* for the time being, but does not affect the conclusions reached here which depend on changes in urine output rather than on absolute figures.

TABLE I

Clinical data in cystinuric subjects *

Patient	Sex	Age in years	Surface area in *sq. meters*	Blood pressure, *mm. Hg*	Blood urea, *mg./100 ml.*	Cystine excretion, *mg./24 hours*	Cystine lysine pattern of amino-acids in urine (by paper chromatography)	Nephrectomy	Renal calculi
G. M.	F	8	0.87	105/75	48	480	Yes	None. Right kidney non-functional	Bladder stone removed 18 months earlier
J. H.	M	27	1.92	130/80	36	1,026	Yes	None	None
A. La.	F	29	1.44	118/80	27	756	Yes	None	Bladder stone removed one month earlier
S. L.	M	38	1.80	130/85	30	1,075	Yes	Right nephrectomy 12 years earlier	Stones removed left kidney 7 years earlier
A. L.	M	38	1.68	160/100	90	645	Yes	None	Bilateral renal and ureteric stones
G. S.	M	39	1.76	175/115	30	1,212	Yes	None	Bilateral renal calculi
P. G.	M	40	1.87	155/115	36	840	Yes	None	Stones present left kidney, removed from right kidney

* All stones removed surgically were shown to be of cystine on analysis.

TABLE II

Control observations (no amino-acid fed) on normals and on cystinuric patients

Subject	Cystine estimation	Fasting	Time in hours ½	1	2	3	4	5	6	7
B. S.	Plasma, *μg./ml.*	12.8	12.8	12.6	11.7		12.0		12.0	
Normal	Urine, *μg./min.*	26.9 ←	33.0	→ : ← 26.2	→ : ←		24.0	→ : ←	24.0	→ :
	Clearance, *ml./min.*	←	2.6	→ : ← 2.15	→ : ←		2.0	→ : ←	2.0	→ :
C. G.	Plasma, *μg./ml.*	11.4	11.7	12.0	10.8		11.7		10.8	
Normal	Urine, *μg./min.*	27.4 ←	31.0	→ : ← 24.0	→ : ←		23.0	→ : ←	21.0	→ :
	Clearance, *ml./min.*	←	2.65	→ : ← 2.25	→ : ←		2.0	→ : ←	2.0	→ :
J. M. W.	Plasma, *μg./ml.*	13.8	11.4	12.0	14.4		14.2		9.0	
Normal	Urine, *μg./min.*	36.5 ←	43.0	→ : ← 32.0	→ : ←		32.0	→ : ←	30.0	→ :
	Clearance, *ml./min.*	←	3.45	→ : ← 2.2	→ : ←		2.25	→ : ←	3.3	→ :
A. La.	Plasma, *μg./ml.*	3.8	5.5	6.0	6.0		4.8		7.2	
Cystinuric	Urine, *μg./min.*	540.0 ←	532.0	→ : ← 512.0	→ : ←		510.0	→ : ←	469.0	→ :
	Clearance, *ml./min.*	←	97.0	→ : ← 85.0	→ : ←		104.0	→ : ←	65.0	→ :

RESULTS

1. *Control studies on normal and cystinuric subjects*

These results are given in Table II. Although there were fluctuations in both blood levels and urine outputs over the seven-hour fasting period these were of small degree. Even though the plasma levels in the cystinuric patient were lower than in the normal subjects, she excreted almost twenty times more cystine. The renal clearance of cystine was between 2 and 4 ml. per min. in the normals and about 90 ml. per min. in the cystinuric patient.

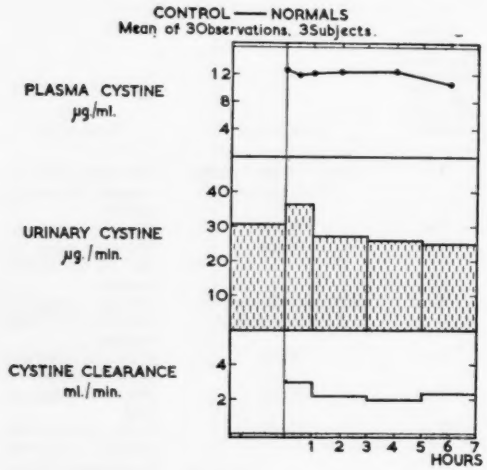

FIG. 1.

The mean results for the three normal subjects are shown in Figure 1. The diagram showing the results obtained in the cystinuric subject is to be found in Figure 7. In these and all subsequent figures and tables basal clearances have not been calculated owing to the fact that the first blood specimens obtained (0 hr.) corresponded in time to the end rather than to the mid-point of the fasting urine collection.

2. *The feeding of l-cystine to normal and cystinuric subjects*

The results are given in Table III and Figures 2 and 3. In the normal subjects there was a small rise in the plasma concentration of cystine and a correspondingly small increase in the urine output; in the cystinuric subject there was a similar but relatively smaller rise. These variations appear to be of doubtful significance as slight fluctuations of this kind also occurred in the control fasting experiments.

That there is no greater increase in cystine plasma level following its ingestion may be due to its prior destruction in the gut by bacteria. The experiment was therefore repeated in two normal subjects after 4.0 g. of aureomycin had been taken in divided doses over the preceding 48 hours. The results were very similar (Table III, expts. 4 and 5). Cystine is extremely insoluble in water at near neutrality and as a result may be absorbed

very slowly from the gut. In an attempt to facilitate absorption 5.0 Gm. of L-cystine was ground in a ball mill with 'Tween 80' as the dispersing agent. The fine emulsion of cystine was then fed to a normal subject and again there was no convincing alteration in the results (Table III, expt. 7).

Although the plasma levels of cystine are lower in the cystinuric than in the normal subjects, they are of the same order of magnitude. The clearance estimations in the case of the normal subjects gave a figure varying betwen 3 and 5 ml. per minute. In the cystinuric patients the much higher clearance figures showed similar variations. These could be explained by the fact that only a single mid-point plasma cystine estimation was done for each one or two-hour clearance period.

The low cystine clearance of G. M. relative to G. S. and A. L. is presumably related to her smaller size.

3. *Feeding of methionine to normal and cystinuric subjects*

The results are shown in Table IV and Figures 4 and 5. In the case of the normal subjects there was a slight increase in both the plasma level and urinary output of cystine. This change was simi-

TABLE III

5.0 g. l-cystine; normals and cystinuric patients

Subject	Cystine estimation	Fasting	Time in hours ½	1	2	3	4	5	6	7
B. S.	Plasma, *μg./ml.*	12.6	13.8	14.4	13.0		12.0		12.0	
Normal	Urine, *μg./min.*	23.0 ←	56.0 → : ←	64.0 → : ←	55.0 → : ←	44.0 → :				
	Clearance, *ml./min.*	←	4.1 → : ←	4.9 → : ←	4.6 → : ←	3.7 → :				
J. M. W.	Plasma, *μg./ml.*	13.0	12.6	12.4	14.3		14.4		10.2	
Normal	Urine, *μg./min.*	36.0 ←	39.0 → : ←	42.0 → : ←	41.0 → : ←	35.0 → :				
	Clearance, *ml./min.*	←	3.1 → : ←	2.9 → : ←	2.9 → : ←	3.4 → :				
B. S.	Plasma, *μg./ml.*	7.8	9.6	13.2	12.6		13.4		11.4	
Normal	Urine, *μg./min.*	30.0 ←	50.0 → : ←	43.0 → : ←	46.0 → : ←	42.0 → :				
	Clearance, *ml./min.*	←	5.2 → : ←	3.4 → : ←	3.4 → : ←	3.7 → :				
J. M. W.	Plasma, *μg./ml.*	11.5	15.6	12.6	13.2		15.2		13.2	
Normal*	Urine, *μg./min.*	36.0 ←	34.0 → : ←	31.0 → : ←	42.0 → : ←	41.0 → :				
	Clearance, *ml./min.*	←	2.2 → : ←	2.3 → : ←	2.8 → : ←	3.1 → :				
B. S.	Plasma, *μg./ml.*	10.8	10.7	16.2	14.2		14.4		12.6	:
Normal*	Urine, *μg./min.*	40.0 ←	49.0 → : ←	53.0 → : ←	42.0 → : ←	43.0 → :				
	Clearance, *ml./min.*	←	4.6 → : ←	3.7 → : ←	2.9 → : ←	3.4 → :				
C. H.	Plasma, *μg./ml.*	9.0	10.8	9.0	11.0		9.0		9.0	
Normal	Urine, *μg./min.*	27.0 ←	21.0 → : ←	29.0 → : ←	27.0 → : ←	29.0 → :				
	Clearance, *ml./min.*	←	1.9 → : ←	2.6 → : ←	3.0 → : ←	3.2 → :				
B. S.	Plasma, *μg./ml.*	11.1	16.9	15.0	14.5		10.3		10.6	
Normal†	Urine, *μg./min.*	32.0 ←	47.0 → : ←	66.0 → : ←	46.0 → : ←	44.0 → :				
	Clearance, *ml./min.*	←	2.8 → : ←	4.5 → : ←	4.5 → : ←	4.2 → :				
G. M.	Plasma, *μg./ml.*	7.2	9.0	7.5	7.2		5.4		4.8	:
Cystinuric‡	Urine, *μg./min.*	400.0 ←	450.0 → : ←	450.0 → : ←	354.0 → : ←	246.0 → :				
	Clearance, *ml./min.*	←	51.0 → : ←	63.0 → : ←	66.0 → : ←	51.0 → :				
G. S.	Plasma, *μg./ml.*	7.8	10.2	8.1	7.2		7.0		6.0	
Cystinuric	Urine, *μg./min.*	842.0 ←	983.0 → : ←	771.0 → : ←	771.0 → : ←	754.0 → :				
	Clearance, *ml./min.*	←	96.0 → : ←	104.0 → : ←	110.0 → : ←	126.0 → :				
A. La.	Plasma, *μg./ml.*	4.4	7.0	5.4	5.4		6.0		4.4	
Cystinuric	Urine, *μg./min.*	540.0 ←	580.0 → : ←	660.0 → : ←	628.0 → : ←	511.0 → :				
	Clearance, *ml./min.*	←	83.0 → : ←	122.0 → : ←	105.0 → : ←	116.0 → :				

* Experiments carried out after the subjects had been on aureomycin 0.5 g. 6-hourly for 48 hours.
† Cystine used in this experiment had been ground in a ball mill with 'Tween 80' as a dispersing agent.
‡ 2.5 g. of l-cystine only given as surface area of patient was only 0.87 sq. meters.

TABLE IV

6.25 g. dl-methionine; normals and cystinuric patients

Subject	Cystine estimation	Fasting	Time in hours ½	1	2	3	4	5	6	7
B. S.	Plasma, *μg./ml.*	9.0	14.4	7.2	10.0		10.2		7.5	:
Normal	Urine, *μg./min.*	19.4 ←	37.5 →	: ←	34.5 →	: ←	26.5 →	: ←	25.5 →	:
	Clearance, *ml./min.*	←	2.6 →	: ←	3.45 →	: ←	2.55 →	: ←	3.4 →	:
J. M. W.	Plasma, *μg./ml.*	9.0	11.4	10.2	9.0		7.8		9.0	
Normal	Urine, *μg./min.*	20.5 ←	33.7 →	: ←	30.0 →	: ←	29.5 →	: ←	25.0 →	:
	Clearance, *ml./min.*	←	3.0 →	: ←	3.3 →	: ←	3.8 →	: ←	2.8 →	:
G. P.	Plasma, *μg./ml.*	5.6	7.2	6.6	10.2		7.2		8.4	
Normal	Urine, *μl./min.*	32.9 ←	43.0 →	: ←	36.5 →	: ←	32.0 →	: ←	27.0 →	:
	Clearance, *ml./min.*	←	6.0 →	: ←	3.6 →	: ←	4.5 →	: ←	3.2 →	:
D. F. E.	Plasma, *μg./ml.*	9.0	8.6	13.2	11.4		10.2		15.6	
Normal	Urine, *μl./min.*	19.4 ←	32.0 →	: ←	35.0 →	: ←	27.0 →	: ←	31.5 →	:
	Clearance, *ml./min.*	←	3.7 →	: ←	3.1 →	: ←	2.6 →	: ←	2.0 →	:
A. L.	Plasma, *μg./ml.*	7.0	9.0	9.6	9.5		11.4		9.7	:
Cystinuric	Urine, *μg./min.*	300.0 ←	270.0 →	: ←	400.0 →	: ←	333.0 →	: ←	366.0 →	:
	Clearance, *ml./min.*	←	30.0 →	: ←	42.0 →	: ←	29.0 →	: ←	38.0 →	:
S. L.	Plasma, *μg./ml.*	5.4	8.9	7.8	7.6		9.5		9.0	
Cystinuric	Urine, *μg./min.*	700.0 ←	733.0 →	: ←	802.0 →	: ←	750.0 →	: ←	825.0 →	:
	Clearance, *ml./min.*	←	82.0 →	: ←	106.0 →	: ←	79.0 →	: ←	92.0 →	:
P. G.	Plasma, *μg./ml.*	7.7	7.2	7.8	6.5		11.0		7.2	
Cystinuric	Urine, *μg./min.*	720.0 ←	648.0 →	: ←	594.0 →	: ←	724.0 →	: ←	706.0 →	:
	Clearance, *ml./min.*	←	90.0 →	: ←	88.0 →	: ←	65.0 →	: ←	98.0 →	:

lar to that found after feeding cystine. The cystinuric subjects showed only small and inconstant variations.

The results in Table IV show that A. L., who had severe impaired renal function (blood urea 90 mg. per 100 ml.), excreted rather less than half the amount of cystine than did S. L. his identical twin. A. L. subsequently died in uremia and showed a progressive diminution of cystine output which paralleled the degree of renal failure.

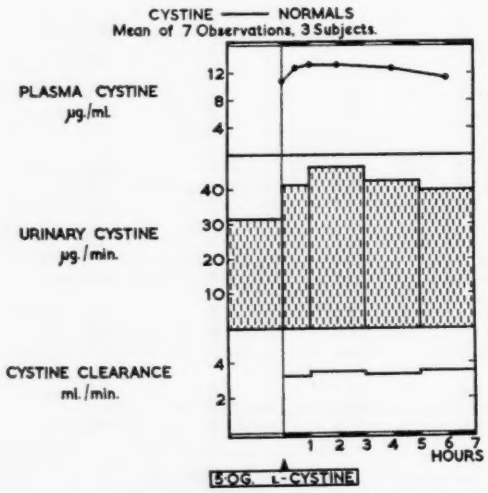

FIG. 2.

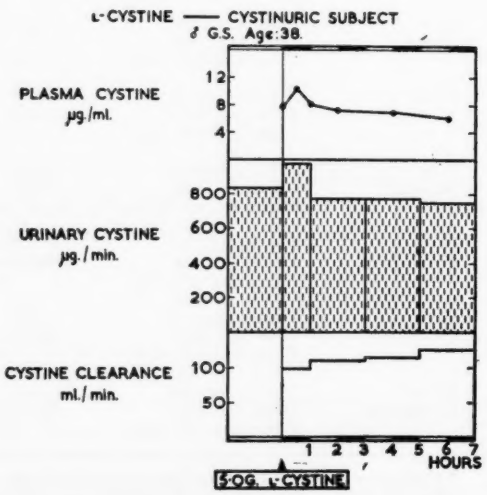

FIG. 3.

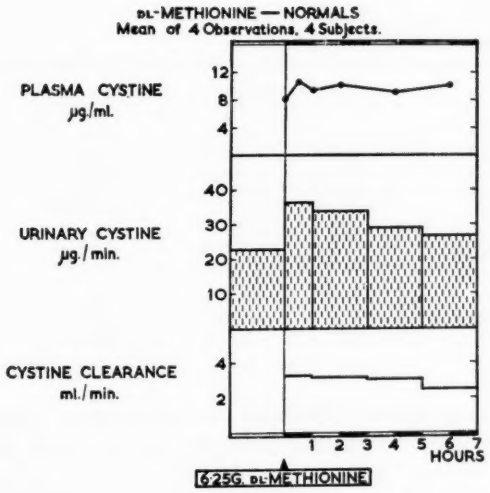

FIG. 4.

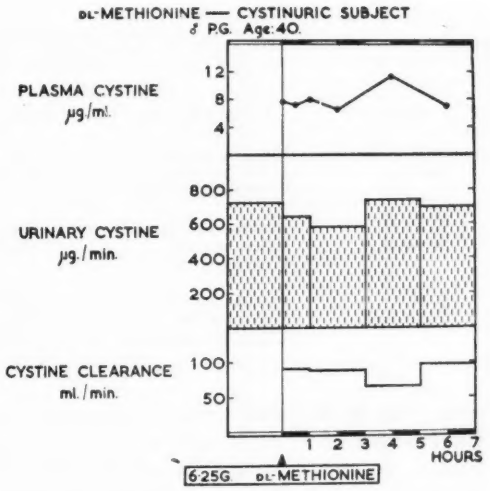

FIG. 5.

4. *Feeding of l-cysteine to normal and cystinuric subjects*

The results are shown in Table V and Figures 6 and 7.

Cysteine produced a very marked increase in the plasma concentration of cystine and a sharp rise in the urinary output. This was seen in both normal and cystinuric subjects. The normal subjects showed an increase in the cystine clearance, which rose in one subject from the basal level of 2 to 4 ml. per min. to a peak of 8.9 ml. per min. The maximum reabsorptive capacity of the tubules for cystine was therefore not exceeded despite a threefold increase in the plasma level. Both cystinuric patients, in spite of the even larger rise in plasma level, did not show a rise in clear-

TABLE V

Cysteine HCl 6.0 g.; normals and cystinuric patients

Subject	Cystine estimation	Fasting	Time in hours ½	1	2	3	4	5	6	7
J. M. W.	Plasma, *μg./ml.*	7.2	14.4	15.0	24.0		14.8		9.6	
Normal	Urine, *μg./min.*	31.0 ←	73.0 → : ←		130.0 → : ←		105.0 → : ←		57.0 → :	
	Clearance, *ml./min.*	←	5.0 → : ←		5.4 → : ←		7.1 → : ←		5.9 → :	
B. S.	Plasma, *μg./ml.*	8.4	25.2	27.0	22.2		19.8	:	10.8	
Normal	Urine, *μg./min.*	32.5 ←	73.0 → : ←		129.0 → : ←		110.0 → : ←		52.5 → :	
	Clearance, *ml./min.*	←	2.9 → : ←		5.8 → : ←		5.5 → : ←		4.9 → :	
T. G.	Plasma, *μg./ml.*	11.4	27.2	27.0	26.2		13.8		13.8	
Normal	Urine, *μg./min.*	28.4 ←	67.5 → : ←		135.0 → : ←		176.0 → : ←		65.6 → :	
	Clearance, *ml./min.*	←	2.5 → : ←		5.1 → : ←		5.5 → : ←		4.8 → :	
G. F.	Plasma, *μg./ml.*	12.0	—	20.4	20.4		15.0		12.0	
Normal	Urine, *μg./min.*	29.8 ←	85.0 → : ←		155.0 → : ←		133.0 → : ←		78.0 → :	
	Clearance, *ml./min.*		— ←		7.6 → : ←		8.9 → : ←		6.5 → :	
A. La.	Plasma, *μg./ml.*	7.4	36.0	38.4	37.0		25.8		12.8	
Cystinuric	Urine, *μg./min.*	580.0 ←	3067.0 → : ←		5000.0 → : ←		2533.0 → : ←		1725.0 → :	
	Clearance, *ml./min.*	←	85.0 → : ←		135.0 → : ←		98.0 → : ←		135.0 → :	
A. L.	Plasma, *μg./ml.*	10.2	25.2	42.0	43.0		24.6		15.6	
Cystinuric	Urine, *μg./min.*	297.0 ←	696.0 → : ←		1108.0 → : ←		785.0 → : ←		475.0 → :	
	Clearance, *ml./min.*	←	27.5 → : ←		26.0 → : ←		32.0 → : ←		30.5 → :	

TABLE VI

Racemic ('dl') cystine 5.0 g. normals and cystinuric patient

Subject	Cystine estimation	Fasting	Time in hours ½	1	2	3	4	5	6	7
J. M. W.	Plasma, *μg./ml.*	9.0	10.2	13.8	12.6		13.8		12.2	
Normal	Urine, *μg./min.*	26.0 ←	213.0 → :		← 394.0 → :		← 304.0 → :		← 285.0 → :	
	Clearance, *ml./min.*	←	21.0 → :		← 31.0 → :		← 22.0 → :		← 23.0 → :	
C. P. H.	Plasma, *μg./ml.*	14.4	13.2	20.2	21.6		18.6		11.4	
Normal	Urine, *μg./min.*	39.0 ←	297.0 → :		← 545.0 → :		← 330.0 → :		← 280.0 → :	
	Clearance, *ml./min.*	←	22.0 → :		← 25.0 → :		← 18.2 → :		← 24.5 → :	
J. H.	Plasma, *μg./ml.*	7.0	7.3	9.0	7.2		6.0		8.1	
Cystinuric	Urine, *μg./min.*	720.0 ←	720.0 → :		← 720.0 → :		← 730.0 → :		← 740.0 → :	
	Clearance, *ml./min.*	←	99.0 → :		← 100.0 →		← 121.0 → :		← 93.0 → :	

ance. As in the methionine experiments, the lower output of A. L. is attributed to his renal failure. In each cystinuric subject the clearance figures were similar to those obtained previously on the same subject when different amino-acids were fed (A. La. Table II, expt. 4; A. L. Table IV, expt. 5).

5. *Feeding of racemic DL-cystine to normal subjects and to one cystinuric subject*

The results appear in Table VI and Figure 8.

In the case of the two normal subjects there was a small rise in the plasma level of cystine. As the percentage rise was exceeded by one normal subject (B. S.) after feeding L-cystine it is probably of no special significance. In spite of these small changes in plasma cystine the cystine output in the urine increased ten-fold. The clearance values therefore showed a like increase. By contrast the results in the cystinuric subject were essentially the same as those seen when L-cystine was fed to other cystinuric patients (Table III), both the output and the clearance remaining virtually constant.

6. *Feeding of L-arginine HCl, L-lysine HCl and Dl-ornithine HCl to a normal subject and to an "incomplete cystinuric" subject*

The normal control (Table VII a and b) showed slight variations in the blood and urine levels of a degree comparable to that obtained when water

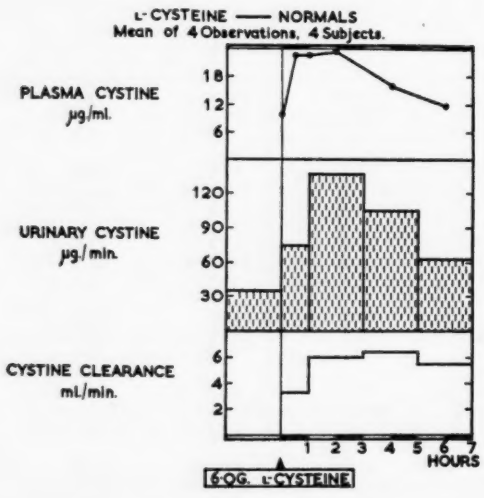

FIG. 6.

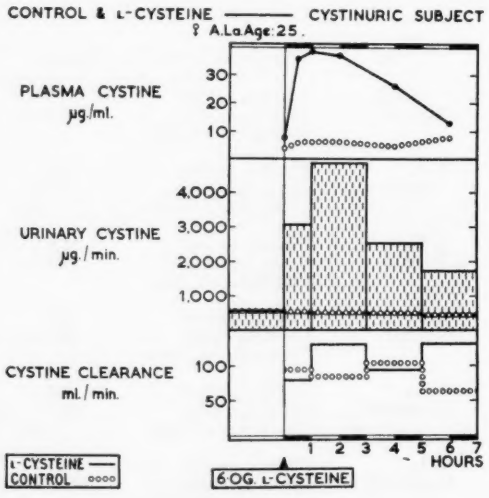

FIG. 7.

TABLE VII

Effect of amino-acid loading on cystine clearance

Subject	Cystine estimation	Fasting	Time in hours ½	1	2	3	4	5	6	7
(a) Arginine HCl 5.0 g., lysine HCl 5.0 g., and ornithine HCl 5.0 g.: normal and "incomplete cystinuric" subject										
J. M. W. Normal	Plasma, *μg./ml.*	9.0	9.6	8.4	10.8		9.2		9.0	
	Urine, *μg./min.*	25.0 ←	38.5 → :	←	29.0 → :		← 27.0 → :		← 32.0 → :	
	Clearance, *ml./min.*	←	4.0 → :	←	2.7 → :		← 2.9 → :		← 3.6 → :	
A. W. "Incomplete cystinuric"	Plasma, *μg./ml.*	12.0	11.9	12.0	13.8		13.2		12.1	
	Urine, *μg./min.*	110.0 ←	168.0 → :	←	150.0 → :		← 92.0 → :		← 96.0 → :	
	Clearance, *ml./min.*	←	14.0 → :	←	11.0 → :		← 7.0 → :		← 7.9 → :	
(b) Glycine 15.0 g.; "incomplete cystinuric" subject										
A. W. "Incomplete cystinuric"	Plasma, *μg./ml.*	10.8	10.6	11.6	12.0		10.8		10.7	
	Urine, *μg./min.*	85.0 ←	85.0 → :	←	86.0 → :		← 77.0 → :		← 77.5 → :	
	Clearance, *ml./min.*	←	8.0 → :	←	7.2 → :		← 7.1 → :		← 7.0 → :	

only was taken by the same subject in an earlier experiment (Table II, expt. 3). It is noted that the clearance values showed no alteration. When fed arginine, lysine, and ornithine the "incomplete cystinuric" subject showed a slight (50 per cent) rise in the excretion of cystine, and an even smaller change in the plasma level. To see whether this small increase of excretion was simply a result of the large amino-acid intake, 15.0 Gm. of glycine were fed to the same subject. The plasma cystine level remained constant as did the urinary excretion.

7. *Simultaneous cystine and inulin clearances; one cystinuric subject*

Three twenty-minute clearances were performed. The results presented in Table VIII show that the clearance values for cystine and for inulin are virtually identical.

8. *Fasting plasma cystine levels in normal and cystinuric subjects*

Fifty plasma cystine levels were measured on 17 fasting normal subjects, and 31 on 9 fasting cystinuric subjects. Cystinuric subjects having marked renal damage were excluded as we have some evidence (unpublished) that the plasma cystine level tends to rise slightly as the output falls with the onset of uremia.

TABLE VIII

	Period 1 *ml./min.*	Period 2 *ml./min.*	Period 3 *ml./min.*
Cystine clearance	95	99	95
Inulin clearance	94	102	104

The normal subjects had a mean fasting plasma cystine level of 10.64 μg. per ml., S.D. 1.87, and the cystinuric subjects a mean of 5.88 μg. per ml., S.D. 1.5. This difference is statistically significant at the 0.1 per cent level.

DISCUSSION

In both normal and cystinuric subjects, L-cysteine alone, of the three sulphur-containing amino-

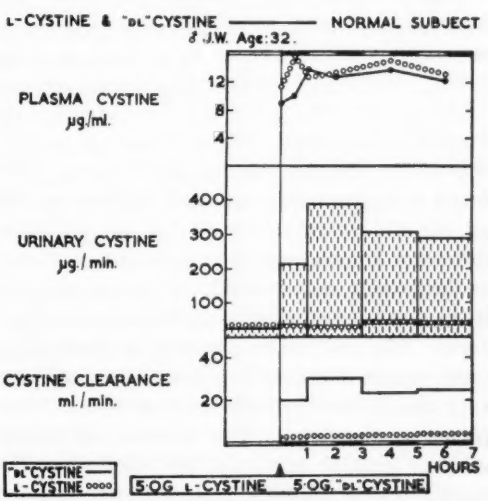

FIG. 8.

acids, produced a large increase in plasma level and urinary output of cystine. (Somewhat higher peak plasma levels were obtained in cystinuric patients). This is presumably because cysteine, being very soluble, can be rapidly absorbed and converted into cystine. Cystine is very insoluble and therefore may be only slowly absorbed. Methionine though readily absorbed (1) is only slowly and in part converted into cystine. In every case there were similar trends between the changes in normal and cystinuric subjects. These results are very similar to those obtained by quite different methods of analysis by Dent, Heathcote, and Joron (1) and serve to reinforce strongly their conclusion that the excessive cystine excretion in cystinuria is the result of a lowered renal threshold for cystine and is not due to a disorder of intermediary metabolism.

Our results after feeding methionine to cystinuric patients are different from those reported by previous workers (13, 14) who consistently found an increase in cystine output. It is possible that the different conditions of our experiments were responsible. In some experiments (unpublished) in which the urine collection was made, as in their series, over a 24 hr. period we have observed increases of 200 to 250 mg. over the basal output. This may have been due to the longer duration of urine collection or to the concomitant ingestion of food.

Further support for the renal hypothesis has been obtained in the more accurately quantitative experiments reported here. In the first place the fasting plasma cystine levels of cystinuric subjects (5.88 μg. per ml., S.D. 1.5) were significantly lower than in normal subjects (10.64 μg. per ml. S.D. 1.5). Fowler, Harris, and Warren (15) found a similar trend, cystinuric subjects av. 6.9 μg. per ml., normal subjects 8.2 μg. per ml. This difference however was not considered significant as their series was too small. In the second place it has now been possible to calculate cystine clearances. The endogenous clearance is about thirty times greater in cystinuric than in normal subjects even though the cystinuric subjects have a lower plasma cystine level. The clearance in normal subjects remains relatively low even when the plasma cystine has been raised after the cysteine feedings. The cystine clearances in cystinuric subjects are of the order to be expected of the glomerular filtration rates especially when allowance is made for the small surface area of G. M. and impaired renal function of A. L. These clearances did not rise as they did in the normal patients when the blood level of cystine was raised after taking cysteine. In the cystinuric J. H. the simultaneous inulin and endogenous cystine clearances were virtually identical over three separate clearance periods. It seems almost certain then that in this patient at least the kidney tubule was unable to reabsorb any of the cystine passing into the glomerular ultrafiltrate.

There is a further argument in support of this that can be derived from the experiments in which DL-cystine was fed. In the normal subjects, though there were but minor changes in the plasma level a marked increase in the output of cystine occurred and the clearance values rose from a basal level of 2 to 4 ml. per min. to 25 to 30 ml. per min. Solubility studies (unpublished) on the urine of C. P. H. obtained after feeding DL-cystine, showed that the major part of the cystine present must have been in a form other than L-cystine. The normal tubule probably absorbs D-cystine less readily (if at all) than L-cystine. In the cystinuric on the other hand no change in cystine clearance occurred after feeding DL-cystine presumably because the tubule cannot reabsorb cystine in any isomeric form, all filtered cystine being excreted.

We would emphasize the importance of making sure that a given batch of cystine is exclusively in the L-form before using it for studies of the kind described here, since most of the cystine on the market is prepared by acid hydrolysis of protein, and some racemization must occur during this process, and will contaminate the final product when insufficiently recrystallized. Albanese (16) has determined cystine outputs after feeding L- and DL-cystine and claimed a large increase after DL-cystine but that no increased output occurred after L-cystine. We have recalculated his figures to express the cystine output in μg. per min. instead of as cystine output per urine sample (30 to 120 min.). The results showed a ten-fold increase after DL-cystine and fourfold increase after L-cystine. Possibly his L-cystine was partly racemized since the latter figure is considerably higher than we would expect and corresponds to our results

(unpublished) with one batch of cystine which was later shown by solubility studies to be partly racemized.

Other workers have shown that the normal kidney tubule distinguishes D- from L-amino-acids (17, 18). The latter suggested (18) on the basis of clearance studies that some D-amino-acid passively diffused through the tubule back into the blood stream. Our results do not suggest any passive diffusion of D-cystine in cystinuric subjects.

The changes in plasma level following the feeding experiments with DL-cystine are of interest. They were similar to those found in a few normal subjects after feeding L-cystine. Silber, Seeler, and Howe (17) using dogs determined plasma α-aminonitrogen levels after intravenous infusions of amino-acids where either 5 per cent or 25 per cent were in D-form. They found similar changes in levels in each case. These experiments suggest that the tissues do not appear to distinguish between D- and L-amino-acids in contrast to their very different handling by the renal tubule.

In cystinuria the urine contains large quantities of lysine, arginine, and ornithine as well as of cystine. Dent and Rose (2) suggested on the basis of similarities in their structure, that there might be a common mechanism responsible for their reabsorption by the tubule. If so it should be possible to demonstrate competition between these substances so that overloading the tubule with lysine, arginine, and ornithine should interfere with cystine reabsorption and therefore increase cystine output and clearance. This experiment was not attempted on a cystinuric subject because we now believe that these subjects have no cystine reabsorption at all. It was, therefore, carried out on a normal subject and on an "incomplete" cystinuric subject (A. W.), the latter being potentially more promising since there was already a decreased ability to reabsorb cystine. The normal subject showed no significant increase in cystine clearance after large oral doses of these three amino-acids. The incomplete cystinuric subject (Table VIIa) showed a small rise in clearance under the same conditions. Probably the normal subject gave a negative result because of the high reserve capacity of the tubule for cystine reabsorption. However the clearance rise of the incomplete cystinuric subject may be interpreted, it was probably not due to unspecific amino-acid competition as a large dose of glycine given to him on another occasion produced no change in cystine clearance (Table VIIb). The differences in clearance, however, were too small to prove conclusively the hypothesis of tubular competition.

In conclusion it can now be stated that stone formation in cystinuric subjects is the immediate result of the defect in cystine reabsorption which resides in the kidney, and contrasts therefore with the stone formation of, for example, hyperparathyroidism, where a primary cause of the hypercalcuria is known to be remote from the renal tract. The therapeutic implications arising from this are being investigated and form the subject of a further communication (19).

SUMMARY

1. Polarographic determinations of cystine in plasma and urine were carried out in normal and in cystinuric subjects before and after feeding cystine, cysteine, and methionine.

2. After feeding cysteine large rises in plasma cystine level occurred, these changes being similar in both normal subjects and in cystinuric subjects. The urine cystine output increased greatly in both groups, being at all plasma levels very much higher in cystinuric than in normal subjects. The results after feeding cystine and methionine were also similar in both normal and cystinuric subjects, except that lesser changes, if any, in cystine level or excretion occurred.

3. The endogenous cystine clearance in normal subjects was 2 to 4 ml. per min. In cystinuric subjects it was about 30 times higher. The cystine clearance was the same as the simultaneous inulin clearance in the one cystinuric patient investigated in this way.

4. Plasma cystine levels in cystinuric subjects are significantly lower than in normal subjects.

5. The kidney tubule in normal subjects appears to reabsorb racemic cystine less readily (possibly not at all) than L-cystine, in cystinuric subjects no difference in excretion was noted, neither apparently being reabsorbed.

6. It is concluded that cystinuria results from greatly diminished (or absent) cystine reabsorption by the renal tubule. No evidence has been

found for an innate disorder of intermediary metabolism of sulphur-containing amino-acids.

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CEREBRAL HEMODYNAMICS DURING CEREBRAL ISCHEMIA INDUCED BY ACUTE HYPOTENSION [1]

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The extreme dependence of the brain upon its circulation for substrates essential for the maintenance of its metabolic activity is well recognized. A cessation of the cerebral circulation for only a few minutes, as occurs in cardiac arrest, results in irreversible brain damage. The brain, for the most part, is an aerobic organ and its large oxygen demands probably account for its unusual susceptibility to circulatory disturbances.

The oxygen consumed by the normal adult brain (3.5 cc. per 100 Gm. per min.) is extracted from approximately 800 cc. of blood passing through it each minute. The minimum blood flow requirements of the brain, *i.e.*, the rate at which signs of cerebral ischemia become manifest, have not been accurately determined. Previous efforts to estimate the critical rate have been made both in animals (1) and in man (2) but the procedures utilized for the determination of cerebral blood flow were not sufficiently quantitative to warrant definite conclusions.

In order to study this problem, cerebral hemodynamic and metabolic changes were determined before and during acute reductions in arterial pressure induced by drug administration and/or postural adjustments.

MATERIALS AND METHOD

Forty-four patients were studied. The subjects in whom hypotension was drug-induced were separated into the following four groups so that the influence of age and hypertension on the susceptibility of the brain to cerebral ischemia could be determined: Group 1, seven normotensive subjects under 50 years of age; Group 2, ten normotensive subjects over 60 years of age; Group 3, eight patients with essential hypertension; and Group 4, seven patients with malignant hypertension. In seven subjects, the mean arterial pressure was reduced significantly below normal but not to the extent of inducing signs of cerebral ischemia. Five patients with spontaneous postural hypotension were also studied.

Control studies were obtained after the subject had been tilted (head up) 30 to 40 degrees for a period of at least 30 minutes. The subjects in the drug-induced hypotension group were then given a 1 per cent solution of hexamethonium [2] intravenously at a rate of 1 mg. of the ion per minute and carefully observed for signs of cerebral ischemia. In the subjects with spontaneous postural hypotension, control studies were done in the horizontal position, and the subjects were then tilted 30 degrees head up, and the experimental flow was done when they developed signs of cerebral ischemia.

The manifestations of cerebral circulatory insufficiency were rather stereotyped and easily recognized. Sighing, yawning, staring, and confusion, *i.e.*, the inability to follow simple commands, were the first indications of cerebral ischemia. When any of these signs appeared, the hexamethonium injection was promptly discontinued, and the second, or experimental flow was begun. A fairly steady state was maintained during the first few minutes making postural adjustments unnecessary. If during the experimental flow, the patient became either unresponsive or too alert, postural adjustments were rapidly made to restore the desired clinical state. The degree of tilt required was usually not more than 10 to 20 degrees. In this way, the clinical state of the subject could be changed in a matter of seconds from one of alertness to confusion, and for the remainder of the flow, the subject was maintained in the latter state.

Scheinberg and Stead's modification (3) of Kety and Schmidt's procedure (4) for the determination of cerebral blood flow (CBF) was utilized in this study. The gas mixtures utilized were those described by Kety and Schmidt (4). Blood oxygen and CO_2 content were determined by the manometric technique of Van Slyke and Neill (5). Blood pH was determined using a Beckman pH meter and an anaerobic glass electrode at room temperature and corrected for 37 degrees centigrade. The cerebral oxygen consumption ($CMRO_2$), cerebral vas-

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cular resistance (CVR), and cerebral oxygen delivery (CDO_2) were calculated as follows:

$$CMRO_2 \text{ (cc. } O_2\text{/min./100 Gm. of brain)} = \frac{CBF \times (A-V)O_2}{100};$$

$$CVR \text{ (mm. Hg/cc. blood/min./100 Gm. of brain} = \frac{MAP}{CBF};$$

$$CDO_2 \text{ (cc. } O_2\text{/min./100 Gm. of brain)} = \frac{CBF \times \text{arterial } O_2}{100}.$$

TABLE IA

Cerebral hemodynamics in acute hypotension associated with cerebral ischemia *

Young Normotensive Subjects

Age	CBF		CVR		$CMRO_2$		AVO_2		MAP	
	C	E	C	E	C	E	C	E	C	E
46	36.8	23.9	2.4	1.8	2.5	2.7	6.72	11.46	88	44
42	43.6	31.9	2.2	0.8	2.6	2.7	5.90	8.55	94	26
35	40.6	30.2	2.1	1.2	2.6	2.5	6.47	8.27	84	36
39	57.4	38.1	1.3	0.9	2.7	3.4	4.64	9.06	75	35
30	58.2	36.2	1.3	1.1	3.4	3.0	5.83	8.21	78	39
32	38.3	29.1	2.3	1.3	2.7	2.6	7.17	8.83	88	38
35	44.0	29.9	1.9	1.0	3.1	3.3	6.94	11.08	83	29
Mean Values										
37	45.6	31.3†	1.9	1.2†	2.8	2.9	6.24	9.35†	84	35†

* In this and other tables, CBF signifies cerebral blood flow in cc./min./100 Gm. of brain; CVR, cerebral vascular resistance in mm. Hg/cc. blood/min./100 Gm. of brain; $CMRO_2$, cerebral oxygen consumption in cc./min./100 Gm. of brain; AVO_2, arterio-venous oxygen difference in volumes per cent; MAP, mean arterial blood pressure in mm. of Hg.

† Significant difference, $p < .01$.

TABLE IB

Cerebral hemodynamics in acute hypotension associated with cerebral ischemia *

Elderly Normotensive Subjects

Age	CBF		CVR		$CMRO_2$		AVO_2		MAP	
	C	E	C	E	C	E	C	E	C	E
59	41.5	34.8	1.4	0.7	1.8	2.0	4.43	5.62	60	23
85	31.3	26.4	2.6	2.0	1.7	2.1	5.44	7.99	82	54
60	41.6	19.6	2.2	1.5	3.1	2.8	7.36	14.01	93	29
88	40.6	30.8	2.2	0.8	2.3	2.4	5.73	7.84	88	24
75	44.6	23.4	1.9	1.2	3.4	3.1	7.71	13.12	85	27
93	47.7	28.6	1.4	0.6	2.3	2.3	4.75	7.94	68	17
77	64.2	35.6	0.9	0.8	3.6	3.2	5.55	8.91	55	28
72	47.7	31.8	1.8	0.9	2.5	2.8	5.20	8.67	88	28
67	66.2	31.8	1.1	0.9	2.9	3.0	4.40	9.30	70	28
69	57.4	27.4	1.7	1.1	3.3	2.9	5.80	10.67	96	30
Mean Values										
75	48.3	29.0†	1.7	1.1†	2.7	2.7	5.64	9.41†	79	29†

* See Table IA for explanation of abbreviations.

† Significant difference, $p < .01$.

TABLE IC

Cerebral hemodynamics in acute hypotension associated with cerebral ischemia *

Subjects with Essential Hypertension

Age	CBF		CVR		$CMRO_2$		AVO_2		MAP	
	C	E	C	E	C	E	C	E	C	E
56	58.4	28.5	2.2	2.2	2.2	2.6	3.76	8.98	130	64
59	43.9	27.4	2.4	1.1	2.4	2.5	5.45	9.27	105	31
72	48.8	33.9	2.7	2.0	3.4	3.1	7.08	9.10	134	68
65	45.6	30.3	2.2	1.5	3.4	3.2	7.48	10.39	102	45
53	49.1	32.1	2.1	0.7	2.9	2.7	5.97	8.41	103	23
68	43.3	35.4	3.0	1.8	2.8	3.1	6.50	8.68	128	65
65	49.9	17.5	2.1	2.2	2.5	2.1	4.96	11.96	103	38
60	44.4	28.0	2.3	1.5	3.0	2.5	6.78	9.01	102	43
Mean Values										
62	47.9	29.1†	2.4	1.6†	2.8	2.7	6.00	9.48†	113	47†

* See Table IA for explanation of abbreviations.

† Significant difference, $p < .01$.

TABLE ID

Cerebral hemodynamics in acute hypotension associated with cerebral ischemia *

Subjects with Malignant Hypertension

Age	CBF		CVR		$CMRO_2$		AVO_2		MAP	
	C	E	C	E	C	E	C	E	C	E
33	46.0	27.9	3.8	3.4	2.6	2.8	5.66	10.03	173	95
39	59.6	39.0	2.8	2.4	3.5	3.3	5.85	8.51	166	95
41	62.6	39.7	2.8	2.7	3.4	2.9	5.44	7.38	173	106
47	75.9	41.0	2.7	2.8	4.3	3.5	5.72	8.52	203	114
42	58.8	26.2	3.2	2.6	3.6	2.9	6.11	11.21	188	69
42	62.4	38.5	2.9	2.0	3.1	3.0	4.92	7.72	183	78
36	56.9	33.7	2.9	1.9	3.7	3.1	6.56	9.48	166	64
Mean Values										
40	60.3	35.1†	3.0	2.5	3.5	3.1	5.75	8.98†	179	89†

* See Table IA for explanation of abbreviations.

† Significant difference, $p < .01$.

The mean arterial blood pressure (MAP) was obtained directly from the femoral artery by means of a damped anaeroid manometer (6) and also by the usual auscultatory method. Since the subjects were tilted, it was necessary to correct for the hydrostatic pressure differences between the femoral and carotid arteries. This pressure differential was equal to the vertical height between the two arteries which varied somewhat in individual studies. The mean arterial pressures, presented in the tables are the calculated mean carotid pressures. This correction, as well as the fact that the subjects were resting in a tilted position for approximately thirty minutes, probably accounts for the normotensive values in some of the subjects with essential hypertension (Table IC).

TABLE IE

Cerebral hemodynamics in acute hypotension associated with cerebral ischemia *

Subjects with Postural Hypotension

Age	CBF		CVR		$CMRO_2$		AVO_2		MAP	
	C	E	C	E	C	E	C	E	C	E
52	59.1	31.3	2.4	1.8	3.2	2.9	5.42	9.22	143	56
47	52.1	36.5	1.7	1.8	3.0	2.5	5.76	6.75	90	64
79	47.7	32.0	1.3	1.1	2.3	2.9	4.74	8.99	63	36
69	44.9	28.6	3.2	1.9	1.9	2.6	4.23	9.09	144	55
78	75.2	47.3	1.0	0.8	3.4	3.4	4.55	7.21	74	39
Mean Values										
65	55.8	35.1†	1.9	1.5	2.8	2.9	4.94	8.25‡	103	50†

* See Table IA for explanation of abbreviations.
† Significant difference, $p < .05$.
‡ Significant difference, $p < .01$.

Electrocardiograms were taken before, during, and after the experimental procedure in 21 subjects, and the internal jugular venous pressures were recorded before and during the study in 14 subjects. The internal jugular venous pressures were measured by means of a spinal fluid manometer; the zero point was considered to be the needle level.

RESULTS

An analysis of the results obtained in the 37 subjects in whom the mean arterial pressure was reduced to levels at which signs of cerebral ischemia became manifest discloses the following: The mean arterial pressure fell from a mean value of 109 mm. Hg to 48 mm. Hg ($p < .01$). The rate of cerebral blood flow decreased significantly from a mean of 51 to 31.5 cc./100 Gm./min. ($p < .01$). The mean cerebral vascular resistance also fell significantly from 2.2 to 1.5 ($p < .01$). The mean cerebral arteriovenous oxygen difference increased significantly from 5.80 volumes per cent to 9.20 volumes per cent ($p < .01$). Despite definite signs of cerebral ischemia, the cerebral oxygen utilization did not change appreciably from control values during the experimental flow. The CO_2 content of the arterial and cerebral venous blood did not change significantly with a reduction of the mean arterial pressure. Similarly there were no significant changes in the blood pH in eleven of the subjects studied.

Tables I, A through E, present the results of the individual groups. Table II presents data on seven subjects in whom the mean arterial pressure was not lowered sufficiently to induce signs of cerebral ischemia.

Though considerable variation is seen in the level of arterial pressure at which signs of cerebral ischemia became manifest, ranging from mean values of 89 mm. Hg in the malignant hypertensive group to 29 mm. Hg in the normotensive group over 60 years of age, the value for the cerebral blood flow is not appreciably different in the individual groups studied (Tables I, A through E). The mean value for cerebral blood flow in the group of subjects in whom cerebral ischemia was not induced (Table II) is significantly higher than in any of the other groups.

TABLE II

Cerebral hemodynamics in acute hypotension without cerebral ischemia *

Age	Diagnosis	CBF		CVR		$CMRO_2$		AVO_2		MAP	
		C	E	C	E	C	E	C	E	C	E
67	Pneumonia	66.8	38.3	1.2	1.1	3.0	3.3	4.46	8.59	82	44
36	Diabetes	65.7	45.8	1.1	1.2	3.8	3.9	5.78	8.55	72	54
23	Dermatitis	77.2	57.2	1.1	1.2	2.7	2.9	3.47	5.01	87	69
18	Rheumatic fever	57.0	49.5	1.4	1.3	3.4	3.6	6.04	7.24	78	66
66	Hypertension	54.9	32.5	3.1	1.6	2.7	3.2	4.92	9.81	168	53
48	Hypertension	50.6	38.8	2.5	1.8	2.9	3.1	5.79	7.96	128	70
43	Uremia	61.4	44.9	2.2	2.2	1.8	1.9	2.87	4.17	133	98
Mean Values											
43		61.9	43.9†	1.8	1.5	2.9	3.1	4.76	7.33‡	107	65‡

* See Table IA for explanation of abbreviations.
† Significant difference, $p < .01$.
‡ Significant difference, $p < .05$.

The mean cerebral vascular resistance fell significantly in both the young and elderly normotensive subjects (Table IA and IB) and subjects with essential hypertension (Table IC). The cerebral metabolic rate did not change significantly from the control values in any of the individual groups. In all groups presented, the cerebral arteriovenous oxygen difference was significantly increased.

In the 14 subjects studied, the internal jugular venous pressure decreased from 83 to 47 mm. of water with an average decrease of 36. Since this represents a difference of approximately 3 mm. Hg, it would only change the cerebral vascular resistance by 0.1 units.

Of interest was the complete lack of clinical and electrocardiographic evidence of coronary insufficiency in these acute studies, particularly in the elderly subjects, many of whom had abnormal electrocardiograms. The control electrocardiograms in 15 subjects were abnormal—two subjects showed evidence of old posterior myocardial infarction, the rest showed non-specific myocardial changes. When symptoms of cerebral ischemia developed, the average heart rate was 64 per minute, a reduction of 35 per cent.

DISCUSSION

Cerebral blood flow and arterial pressure

It is evident from these studies that despite the subject's age, original level of arterial pressure, per cent reduction of arterial pressure, or original rate of cerebral blood flow, signs of cerebral ischemia developed when the mean cerebral blood flow was reduced to 31.5 cc. per min. per 100 Gm. of brain. The mean values of the experimental cerebral blood flows for the individual groups varied between 29 and 35 cc. per 100 Gm. of brain per min. The fact that in the group without cerebral ischemia (Table II) the subjects were alert despite a mean cerebral blood flow of 43.9 indicates that the critical rate of cerebral blood flow is below this value. Apparently significant reductions in cerebral blood flow from normal are possible without inducing manifestations of cerebral ischemia.

The degree of fall in arterial pressure required to produce cerebral ischemia, as might be anticipated, was related to the cerebral vascular resistance. When the cerebral vascular resistance could be significantly reduced (all groups except the postural hypotensive group and the malignant hypertensive group), the arterial pressure could be lowered markedly before signs of cerebral ischemia became manifest. When the vascular resistance could not be significantly reduced, the level of arterial pressure at which cerebral ischemia developed was higher. Only in situations where the vascular resistance remains fixed, can there be a direct relationship between per cent reduction in cerebral blood flow and mean arterial pressure. Since the mean arterial pressure at which signs of cerebral ischemia developed varied considerably (89 to 29 mm. Hg), it is evident that a critical level of arterial pressure as such does not exist.

Cerebral vascular resistance

The initial values for the cerebral vascular resistance in the individual groups varied. The finding of an increased cerebral vascular resistance in the hypertensive subjects confirms the results of Kety and others (7–10). The highest mean value for the cerebral vascular resistance was noted in the malignant hypertension group. While the increased cerebral vascular resistance in the essential hypertension group was significantly reduced with hypotension, the cerebral vascular resistance in the malignant hypertension group remained relatively fixed. It may be that the inability to overcome this increased cerebral vascular tone accounted for the development of signs of cerebral ischemia at normal levels of arterial pressure.

It was particularly interesting that the elderly normotensive subjects showed a significant reduction in cerebral vascular resistance when their mean arterial pressures were reduced. This confirms previous observations demonstrating considerable alterations in the vascular tone of elderly subjects (11, 12). In the group studied, the cerebral vascular resistance was not as high as we expected it might be. In a preliminary study (13) on elderly hypertensive subjects with a markedly elevated cerebral vascular resistance, signs of cerebral ischemia have occurred at normotensive levels. An overall decrease in cerebral vascular resistance in normotensive and hypertensive subjects with cerebral arteriosclerosis should not be taken to mean that all cerebral vessels are capable of under-

going the same degree of relaxation. It is possible that with an acute drop in blood pressure, circulation through the more rigid cerebral vessels may be seriously impaired. This may explain why transient episodes of cerebral ischemia may sometimes be associated with a relatively slight decrease of the mean arterial pressure. The explanation for the decrease in cerebral vascular resistance attendant with the reduction in mean arterial pressure is not apparent from these studies since there was no change in either pH or CO_2 content.

Cerebral metabolic rate

Even though signs and symptoms of cerebral ischemia were present in most of the subjects studied, there was no significant change in cerebral oxygen utilization between the control and induced hypotension studies. There can be no question that in these acute experiments the manifestations of cerebral ischemia were most likely due to a decreased oxygen delivery to certain cerebral cells since there was neither hypoglycemia nor any obvious impairment of cerebral enzymatic activity. It is suggested from the neurological signs evoked that not all regions of the brain were equally affected by the cerebral ischemia. The variable sensitivity of the different areas of the brain to reduced oxygen tensions and to depressant drugs is well recognized. It may be that with a reduction of the rate of cerebral blood flow those areas with the highest metabolic requirements are first affected. The failure to observe significant changes in cerebral oxygen utilization in these studies may be due to the fact that the method employed is not sufficiently sensitive to reflect changes in the metabolic activity of a relatively small number of cerebral cells. In this regard it should be mentioned that during sleep (14) and with light Pentothal® anesthesia (15) one does not observe significant changes in cerebral oxygen utilization with this procedure.

Cardiovascular effects

The fact that small doses of hexamethonium were given (average dose was 8 mg. of the ion) and the frequent finding of bradycardia and increased perspiration in the drug-induced studies suggests that full blocking doses of the drug were not given, and the effects observed were not entirely drug-induced. The hypotensive effect of small doses of the ganglionic blocking agent enhanced by tilting apparently activated vasovagal reflexes which accounted for the bradycardia observed. With the onset of collapse, cardiac output and right ventricular pressures decreased sharply (16). These latter observations are consistent with the concept that the mechanism of cerebral ischemia was due to loss of peripheral vasoconstrictor tone which in our tilted subjects promoted failure of venous return and hence of cardiac output.

SUMMARY AND CONCLUSIONS

1. The effect of cerebral ischemia induced by the intravenous administration of hexamethonium and/or tilting on cerebral hemodynamics and metabolism was determined in young and elderly normotensive subjects and in subjects with essential and malignant hypertension. Seven subjects with postural hypotension were also studied.

2. In all subjects, signs and symptoms of cerebral ischemia developed when the cerebral blood flow was reduced to 31.5 cc. per 100 Gm. of brain per min. (mean value).

3. The degree of fall in mean arterial pressure which resulted in cerebral ischemia varied from 29 to 80 mm. Hg.

4. Subjects with a relatively fixed cerebral vascular resistance (malignant hypertension) developed cerebral ischemia at higher mean arterial pressures.

5. Although blood pressures were lowered to levels resulting in clinical signs and symptoms of cerebral ischemia, no change in the over-all cerebral oxygen consumption was noted.

6. The development of clinical signs of cerebral insufficiency without simultaneous clinical or laboratory evidence of coronary insufficiency suggests that the brain may be more sensitive to acute hypotension than is the heart.

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THE USE OF HEMOLYSIN INHIBITION IN THE STUDY OF EXPERIMENTAL AND CLINICAL HYPERLIPEMIA: THE NON-SPECIFIC INHIBITION OF STREPTOLYSIN O BY SERUM LIPOPROTEINS[1]

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Streptolysin O is one of a group of hemolytic toxins that have in common the property of being inhibited by cholesterol. This property is also shared by saponin, a hemolytic agent of quite a different type. Other hemolytic toxins, such as streptolysin S, are not inhibited by cholesterol but are neutralized by phospholipids (1–3).

Certain observations suggest that the inhibition of these hemolysins by serum is influenced by the physicochemical state of serum lipoproteins. For example, the cholesterol contained in normal human and animal sera does not inhibit streptolysin O hemolysis. Old samples of sera, or sera contaminated by certain strains of bacteria, do become strongly inhibitory, however (2). Under these conditions the serum cholesterol is presumably liberated from lipoprotein complexes. Streptolysin S has been shown to be inhibited by serum lipoproteins. This inhibition may be varied by measures which alter the native state of these proteins or by diseases which are associated with disturbances in lipid metabolism (4–8). Also, the inhibition of saponin hemolysis by whole plasma is not a simple function of the concentration of its lipid components. The sum of the inhibition produced by each of the lipid and protein components of plasma is not as great as the inhibition of saponin hemolysis by whole plasma (9).

These and other observations suggest that further study of hemolysin inhibition by sera of abnormal lipid composition may detect changes in lipoproteins in intact serum which may not be readily revealed by conventional analytical methods. This report describes the inhibition of two cholesterol sensitive hemolysins, streptolysin O and saponin, by the sera of patients with disturbances of lipid metabolism.

MATERIALS AND METHODS

Preservation of sera: Sera obtained from patients and from animals were stored at −20° C. Determinations of inhibition of streptolysin O were carried out before freezing the serum, and within 48 hours after blood had been drawn. It was observed that freezing and thawing of samples resulted, in some instances, in significant loss of inhibition or in bizarre "prozone" reactions.

Titrations and chemical determinations: Titration of *streptolysin O inhibition* (*SOI*) was performed by the following method suggested by Dr. Alan W. Bernheimer: Dilutions of serum and of streptolysin O were made in saline-phosphate buffer at pH 7.0 (M/15 phosphate and M/12.9 sodium chloride). Sera were heated for 30 minutes at 56° C. and two-fold serial dilutions were prepared in volumes of 0.5 ml. per tube. When more exact titration was required, closer dilution increments of 0.1 in the logarithm of the dilution factor were employed. A partially purified and concentrated preparation of streptolysin O containing 8,000 hemolytic units per ml. was used in these studies.[2] (A hemolytic unit is defined as the amount of streptolysin O in one ml. of saline-phosphate buffer which produces 50 per cent hemolysis of 1 ml. of a 0.7 per cent suspension of washed, human erythrocytes in 30 minutes at 37° C.) One ml. of a 1:5 dilution of the preparation of streptolysin O employed was reduced by the addition of one ml. of a 1 per cent solution of cysteine brought to pH 7 by the addition of 1 normal sodium hydroxide with bromthymol blue as indicator. The streptolysin O-cysteine solution was held for 10 minutes at 20° C. to allow complete reduction of the hemolysin to take place. The solution of streptolysin O was further diluted to a final concentration of 1:250 in saline-phosphate buffer to obtain a final dilution containing 32 hemolytic units per ml. of active, reduced streptolysin O. This solution was promptly added in a volume of 0.5 ml. to each tube containing 0.5 ml. of serum dilution and the mixture incubated at 20° C. for 10 minutes. One ml. of a 0.7 per cent suspension of washed, human erythrocytes was added to each tube and the tubes were incubated for

[1] This study was supported in part by a grant from the Masonic Foundation for Medical Research and Human Welfare.

[2] Kindly supplied by Dr. Alan W. Bernheimer.

30 minutes at 37° C. After this incubation the tubes were centrifuged for five minutes at 1,500 R.P.M. in a No. 2 International centrifuge and the degree of hemolysis in the supernate of each tube was noted. The endpoint was considered the highest dilution of serum causing complete inhibition of hemolysis. With each group of unknown sera, a standard serum of known titer was run as a control. The number of units of streptolysin O inhibition per ml. of serum was expressed as the reciprocal of the quantity of serum producing complete inhibition of hemolysis. The unitage thus expressed is approximately similar to the combining unit of Todd (10) employed for the measurement of antistreptolysin O antibody.

Antistreptolysin O determinations for the measurement of specific serum antibody (in distinction to the above measurement of non-specific streptolysin O inhibition by lipids) were performed by the method of Todd (10) as modified by Rantz and Randall (11).

Saponin inhibition was measured by a method identical to that described above for streptolysin O inhibition, substituting for streptolysin O a 10 per cent aqueous solution of saponin diluted 1:80 in saline-phospate buffer. This solution contained 16 hemolytic units per 0.5 ml. *Streptolysin S inhibition* was determined by the method of Stollerman and Bernheimer (6).

Serum cholesterol concentration was determined by the method of Schoenheimer and Sperry (12) as modified by Sperry and Webb (13). Serum phospholipid concentration was measured by the method of Zilversmit and Davis (14). Lipid was removed from serum at low temperatures with ether-alcohol mixtures as described by MacLeod and Avery (15).

Absorption of specific antibody against streptolysin O: to differentiate specific from non-specific inhibition of streptolysin O by human sera, antistreptolysin O antibody was absorbed by incubating equal parts of serum and streptolysin O solution, containing an excess of the hemolysin, for 15 minutes at 37° C. The residual, unneutralized streptolysin O was then destroyed by heating the mixture at 56° C. for one hour.

Digestion of serum phospholipid by lecithinase: The lecithinase employed was the alpha toxin of *Clostridium welchii,* partially purified according to the method of Adams and Hendee (16). The lot employed contained 250 MLD for mice per ml. and 8 Lb units (antitoxin binding power). Digestion of serum lecithin was carried out as described by Stollerman, Bernheimer, and MacLeod (4).

Methods for inducing hyperlipemia in rabbits

Cholesterol feeding: Six rabbits weighing 1.5 to 3.0 Kg. consumed an average of 34 Gm. of cholesterol per week. Purina rabbit pellets were coated with cholesterol by soaking them in ether in which cholesterol had been dissolved and then allowing the ether to evaporate. Rabbits were bled from an ear vein at appropriate intervals.

Heparin was administered to a group of six rabbits which were also fed cholesterol as described above. Intravenous injections were made three times weekly of 2.5 ml. of a solution containing 10 mg. of heparin per ml. (Lederle's aqueous heparin) during the period of cholesterol feeding.

Triton WR-1339,[3] a non-ionic detergent, was administered intravenously three times weekly to a group of six rabbits in doses of 1.25 ml. per Kg. of a 12.5 per cent aqueous solution (17). While receiving Triton the animals were fed a diet of regular Purina rabbit pellets without added cholesterol.

Alloxan diabetes was induced in four rabbits by minor modifications of the method employed by Pierce (18). Four weeks after permanent diabetes had been established, cholesterol was fed as described above. Several rabbits which developed transient acute hyperlipemia within a few days of receiving an injection of alloxan were bled at frequent intervals until the hyperlipemia cleared or the animal died of alloxan toxicity. The development of diabetes was observed by repeated determinations of blood sugar concentrations.

RESULTS

Hemolysin inhibition by hyperlipemic rabbit sera

Cholesterol-fed rabbits: Determinations of titers of streptolysin O inhibition (SOI), streptolysin S

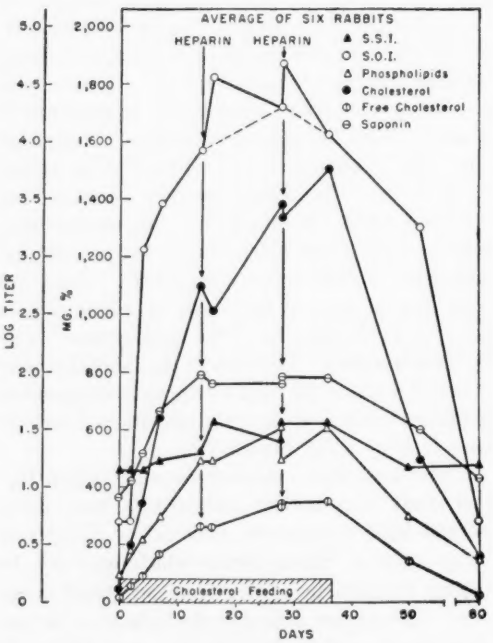

FIG. 1. EFFECT OF CHOLESTEROL FEEDING UPON STREPTOLYSIN O, STREPTOLYSIN S AND SAPONIN INHIBITION BY THE SERA OF RABBITS

[3] Kindly supplied by Rohm and Haas.

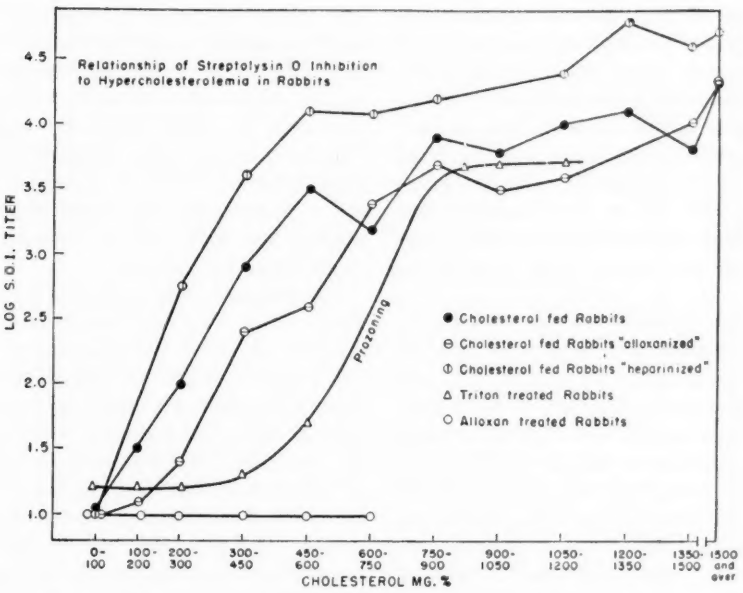

FIG. 2. RELATIONSHIP OF STREPTOLYSIN O INHIBITION TO VARIOUS TYPES OF EXPERIMENTAL HYPERCHOLESTEROLEMIA IN RABBITS

inhibition (SSI) saponin inhibition, total and free cholesterol and total phospholipid levels were made on sera obtained from serial bleedings of rabbits fed cholesterol. When the total cholesterol concentration in serum approached levels of approximately 250 mg. per cent, there was an abrupt and striking rise in SOI. Streptolysin O inhibition increased to levels of over 10,000 units per ml. and in some animals to 100,000 units per ml. There was also a rise in saponin inhibition titer but of a much smaller magnitude. Streptolysin S inhibition increased two to four fold (Figure 1).

The marked streptolysin O inhibition disappeared completely when serum lipids were extracted at low temperatures with alcohol-ether mixtures. In contrast, the streptolysin O inhibition was unaffected by absorption of the serum with streptolysin O. This clearly differentiated the inhibition due to lipids from that due to specific antibody. The contrasting effect of these two procedures upon sera of cholesterol-fed rabbits and sera of patients with high titers of specific antistreptolysin O antibody is demonstrated by the data in Table I.

Effect of intravenous injection of heparin: Because of reports of alterations in the physical state of serum lipids following intravenous injection of heparin (19) the effect of heparin upon inhibition of streptolysin O was studied. Hypercholesterolemic, cholesterol-fed rabbits, bled three hours after an intravenous injection of 10 mg. of heparin, showed a two to four-fold rise in the titer of SOI. This occurred in the absence of significant changes in serum concentration of total or free cholesterol or of phospholipid (Figure 1).

TABLE I

Differentiation of non-specific inhibition of streptolysin O by serum lipoproteins from inhibition due to specific antibody

Hypercholesteremic rabbits	Streptolysin "O" inhibition, u/cc. (non-specific inhibition)		
	Whole serum	"Defatted" serum	Absorbed* serum
No. 60	6,400	<10	6,400
No. 80	19,200	<10	19,200
Rheumatic fever patients	**Antistreptolysin "O" titer, u/cc. (specific inhibition)**		
	Whole serum	"Defatted" serum	Absorbed* serum
M. G.	333	250	<50
J. K.	333	250	<50

* Absorbed with streptolysin O.

Determinations of SOI made on the sera of an additional group of six rabbits fed cholesterol and receiving intravenous injections of heparin three times weekly showed consistently higher SOI values at comparable serum concentrations of cholesterol than were observed in the sera of rabbits fed cholesterol alone (Figure 2). This enhancement of SOI could not be demonstrated when heparin was added to these hypercholesterolemic sera *in vitro* nor was heparin itself found to be inhibitory.

Effect of administration of alloxan: The transient hyperlipemia which occurs in some animals shortly after administration of alloxan (20, 21) was studied. In contrast to the sera of cholesterol-fed rabbits, the hyperlipemic sera of animals acutely toxic from alloxan failed to inhibit streptolysin O even at a serum cholesterol concentration of 675 mg. per cent. Rabbits which survived alloxan injection and remained diabetic were observed until the transient hypercholesterolemia had cleared (usually about two to three weeks after injection). The rise in serum cholesterol which occurred was now associated with a marked rise in streptolysin O inhibition, comparable to that observed in normal rabbits fed cholesterol (Figure 2).

Effect of intravenous injection of Triton WR 1339: Rabbits which were repeatedly injected intravenously with the non-ionic detergent Triton WR 1339 became progressively hyperlipemic as described by Kellner, Correll, and Ladd (17). The sera of these animals did not inhibit streptolysin O hemolysis until cholesterol concentrations approached a level of approximately 400 to 600 mg. per cent. At these levels a "prozone" effect was noted in which complete inhibition of the hemolysin occurred only at high dilutions of serum. This "prozone" tended to disappear when maximal concentrations of serum cholesterol were reached but could not be completely abolished (Table II and Figure 2).

The prozone effect could not be explained by the formation of a new hemolysin resulting from injection of Triton since incubation of red blood cell suspensions with varying dilutions of serum from Triton-treated animals did not reveal hemolytic activity. The possibility was also considered that incubation of streptolysin O with the serum of Triton-treated rabbits might have resulted in the production of an additional hemolytic substance. This possibility was excluded by experiments wherein streptolysin O was incubated with such sera. After neutralizing excess streptolysin O by the addition of its specific antibody, the mixture was tested and no hemolytic activity was found. Triton WR 1339 when added to these sera *in vitro* was not hemolytic in concentrations of less than 12.5 per cent. It is unlikely, therefore, that the prozone could be explained by the presence in serum of Triton *per se*. Injections of heparin failed to abolish the prozone phenomenon. The mechanism whereby low serum di-

TABLE II

Inhibition of streptolysin O hemolysis by sera obtained during administration of Triton WR 1339, three times weekly, intravenously

	Rabbit No. 78 *Hemolysis inhibition and concentrations of cholesterol and phospholipid, mg. %, in serum obtained on the dates indicated* *					
	9/22	9/25	10/16	10/30	11/13	12/11
Serum dilutions	Chol. 33 Phos. 79	Chol. 268 Phos. 500	Chol. 494 Phos. 918	Chol. 677 Phos. 1452	Chol. 938 Phos. 1932	Chol. 1311 Phos. 2616
1/10	++++	++++	++++	++	+	++++
1/20	++++	+++	++++	++	±	+++
1/40	++++	+++	++++	+	±	+++
1/80	++++	++	++++	±	−	++
1/160	++++	++	+++	−	−	±
1/320	++++	+++	++	−	−	−
1/640	++++	+++	+	−	−	−
1/1280	++++	++++	+	−	−	−
1/2560	++++	++++	+	−	−	−
1/5120	++++	++++	+	−	−	±
1/10,240	++++	++++	+	−	±	±
1/20,480	++++	++++	++++	±	+	+
1/40,960	++++	++++	++++	+	++	++

* Complete hemolysis ++++; no hemolysis −.

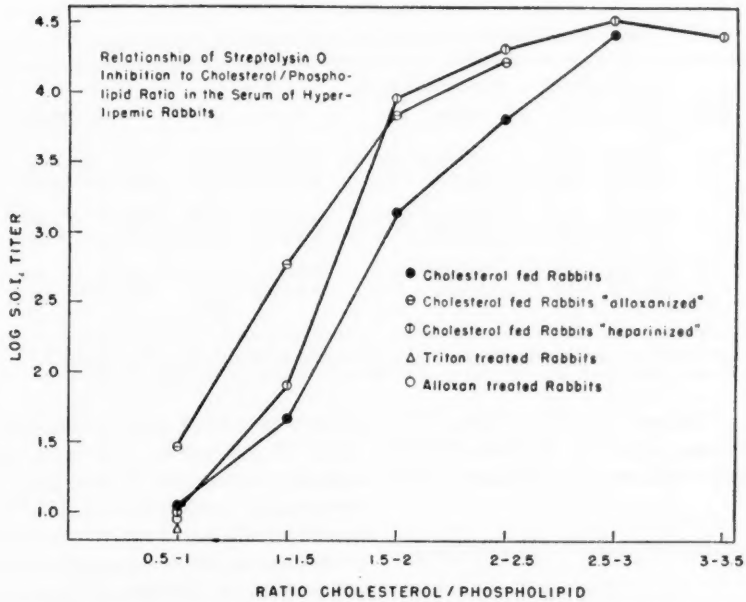

FIG. 3. RELATIONSHIP OF STREPTOLYSIN O INHIBITION TO CHOLESTEROL/PHOSPHOLIPID RATIO IN THE SERUM OF HYPERLIPEMIC RABBITS

lutions of this type of hyperlipemic serum fail to inhibit streptolysin O whereas inhibition is apparent at high dilutions was not explained.

The relationship of streptolysin O inhibition to cholesterol phospholipid ratios in the sera of hyperlipemic rabbits: An attempt was made to relate the differences observed in the SOI of various types of hyperlipemic rabbit sera to the ratio of cholesterol phospholipid concentration. The sera of normal rabbits fed cholesterol, and those which received heparin intravenously while being fed cholesterol, showed strong streptolysin O inhibition when the cholesterol/phospholipid ratio in serum approached 1.5. Rabbits made diabetic with alloxan and fed cholesterol showed a prompt rise in SOI titer when this ratio approached 1.0 and increased as the ratio increased (Figure 3).

The hyperlipemic sera obtained from rabbits during the stage of acute alloxan toxicity, and which failed to inhibit streptolysin O, contained relatively high concentrations of phospholipid. The cholesterol/phospholipid ratio of these sera was considerably below 1.0. Similarly, the sera of animals which received Triton WR 1339 had relatively high concentrations of phospholipid and inhibited streptolysin O only at high serum dilutions. Thus, the inhibition of streptolysin O by rabbit sera appeared to be related in a general way to the ratio of cholesterol to phospholipid (Figure 3).

The inhibition of saponin by hyperlipemic rabbit sera: Sera obtained in the experiments described above were also tested for inhibition of saponin to determine whether this hemolysin, which is also inhibited by cholesterol, was affected in the same fashion as streptolysin O. Several differences were noted. Inhibition of saponin hemolysis by the sera of cholesterol-fed rabbits was roughly proportionate to serum cholesterol concentration. In contrast to the findings with streptolysin O inhibition, intravenous injection of heparin failed to enhance the inhibition of hemolysis by saponin. In addition, the sera of alloxan-toxic animals, and of Triton-treated animals, showed even higher degrees of inhibition than that of cholesterol-fed rabbits at corresponding concentrations of serum cholesterol. In contrast to streptolysin O inhibition, therefore, sera with proportionately higher concentrations of phospholipids were associated with enhancement of in-

TABLE III

Non-specific inhibition of streptolysin O by human hyperlipemic sera

Diagnosis	No. of Patients	Cholesterol, *mg.* %		Phospholipid, *mg.* %		C/P ratio		SOI,* *u/cc.*		Sap. inhib., *u/cc.*	
		Range	Av.	Range	Av.	Range	Av.	Range	Median	Range	Av.
Obstructive jaundice	20	86–495	256.	159–1215	461.	0.42–0.93	0.71	160–<20	<20	10–33	18.
Nephrosis	17	306–1060	554	326–888	554	0.72–1.25	1.02	†640–<10	<10	20–100	54.
Familial hypercholesterolemia	5	208–468	355	260–676	407.	0.48–1.25	0.93	†640–40	40		
Diabetes	2	292–300	296	380–400	390	0.75–0.77	0.76	20–<20	20		
Coronary occlusion	3	200–356	265.	238–319	278.	0.84–1.12	0.98	20–10	10		
Hepatitis	9	146–254	185.	189–572	319.	0.41–0.80	0.62	160–<20	40		
Myxedema	1		418		345		1.21		40		

* SOI determinations were performed after absorption of specific antibody with streptolysin O.
† The serum of one patient in this group had an SOI titer of 640 and a C/P ratio of 1.25.

hibitory activity against saponin (Figure 4). There was no tendency of the sera of Triton-treated rabbits to show "prozoning" reactions with saponin.

Hemolysin inhibition by human hypercholesterolemic sera

The sera of patients with a variety of diseases associated with hypercholesterolemia were studied for inhibition of hemolysis by streptolysin O and saponin. Sera with SOI titers of more than 160 units per ml. were absorbed with streptolysin O to remove specific antibody. Of 57 patients studied, the sera of only two showed significant streptolysin O inhibition (640 units) after specific antibody had been absorbed. One of these two patients manifested the nephrotic syndrome and the other had familial hypercholesterolemia. The ratio of cholesterol/phospholipid in both sera was 1.25, the highest observed in this series (Table III).

With the single exception mentioned above, sera from patients with the nephrotic syndrome showed the characteristic extremely low antistreptolysin O titers that have been previously described (22, 23), and which have been explained in part by loss of specific antibody in the urine (24). Despite the very high values for serum cholesterol encountered in this group of patients, non-specific streptolysin O inhibition was observed in only one of the 17 patients studied.

Because the above data suggest that non-specific streptolysin O inhibition is apparent only in sera in which the concentration of cholesterol significantly exceeds that of phospholipids, an attempt was made to determine whether enzymatic degradation of serum phospholipid would result in enhanced streptolysin O inhibition. The sera of 15 patients with the nephrotic syndrome, and of 15 patients with hypercholesterolemic disorders, were assayed for streptolysin O inhibition before and after digestion with lecithinase (purified alpha toxin of *Clostridium welchii*). Nine of the 15 sera of patients with nephrosis and 7 of the 15 sera of patients with other hypercholesterolemic syndromes, showed striking increase in streptolysin O inhibition following lecithinase digestion. Treatment of these sera with lecithinase resulted in all instances in loss of over 90 per cent of phospholipid.

Total cholesterol content before and after lecithinase digestion remained virtually unaffected.

TABLE IV

Effect of lecithinase treatment upon streptolysin O inhibition of human hyperlipemic sera

Patient	Disease	SOI, *u/cc.*		Phospholipid, *mg.* %		Cholesterol, *mg.* %	
		Control	Lecithinase treated	Control	Lecithinase treated	Control	Lecithinase treated
SIM	Nephrosis	<10	2,560	694	48	685	640
MEN	Nephrosis	<10	20	562	41	604	590
YOR	Ca of liver	80	10,240	1,132	64	454	459
KROP	Xanthomatosis	40	80	406	30	401	384

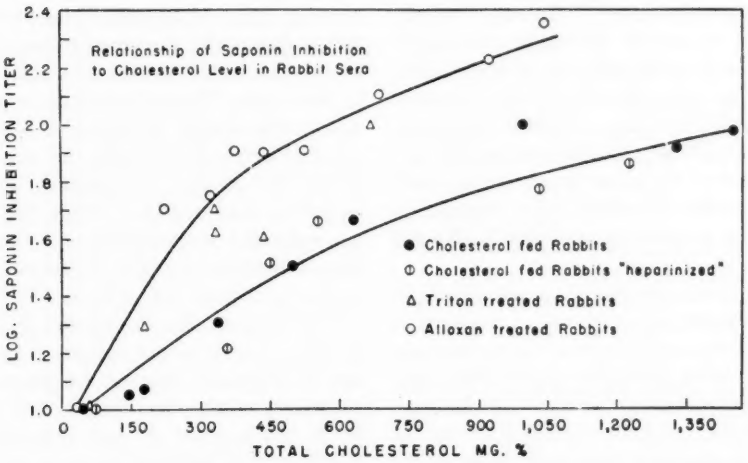

FIG. 4. RELATIONSHIP OF SAPONIN INHIBITION TO CHOLESTEROL LEVEL IN RABBIT SERA

The failure of some sera to develop increased inhibition of streptolysin O was, therefore, not due to either failure of the enzyme to disrupt phospholipid or to destruction of cholesterol (Table IV).

The inhibition of saponin by human hypercholesterolemic sera paralleled, in general, the concentration of serum cholesterol as has been previously observed (9). At normal concentrations of serum cholesterol, saponin inhibition titers ranged from 10 to 16 units. At serum cholesterol levels of 1,000 mg. per cent, saponin inhibition titers were approximately 100 units. It appears that in human, as in rabbit, serum the lipoprotein inhibition of saponin is quite distinct from that of streptolysin O inhibition.

The administration of heparin intravenously in doses of 100 mg. to one patient with hypercholesterolemia due to the nephrotic syndrome and to two patients who suffered recent coronary occlusion and who had high normal values of cholesterol in their sera, failed to produce significant increase in SOI titers. Alimentary hyperlipemia was not associated with increased SOI, even following the "clearing" effect produced by injection of heparin.

Because of reports that the sera of some patients with infectious hepatitis demonstrate marked non-specific streptolysin inhibition (25, 26), 17 sera of six patients with infectious hepatitis [4] were assayed for SOI from serial bleedings made during the course of their illness. None of the sera studied showed significant elevation of SOI titer.

[4] These sera were kindly supplied by Dr. Roderick Murray of the National Microbiological Institute, National Institutes of Health.

DISCUSSION

One of the objectives of this study was to determine whether various types of hyperlipemic sera could be characterized by their capacity to inhibit the hemolysins employed. Marked inhibition of streptolysin O hemolysis is characteristic of the sera of rabbits fed cholesterol and appears to be related to the high ratio of cholesterol to phospholipid in such sera. The "prozone" type of inhibition of streptolysin O by the sera of rabbits injected with Triton WR 1339 is also peculiar to these sera for reasons which are not explained. Human hypercholesterolemic sera do not inhibit streptolysin O hemolysis non-specifically, except in rare instances when the ratio of cholesterol to phospholipid is unusually high. No characteristic pattern of hemolysin inhibition was observed, therefore, by which one clinical type of hyperlipemia could be distinguished from another. In this respect the inhibition of streptolysin O by human sera appears to have limited clinical application.

The capacity of serum cholesterol to inhibit streptolysin O hemolysis may be of some interest, however, as a reflection of the structure and composition of serum lipoproteins. Serum cholesterol is considered to be combined with phospholipids in large lipoprotein complexes of varying size

(27, 28). The configuration of cholesterol within such molecules apparently determines the availability of certain chemical groups to inhibit various hemolysins. The same serum may inhibit saponin hemolysis strongly but may not inhibit streptolysin O hemolysis at all. Cholesterol inhibits the hemolytic action of saponins by combining with them to form double compounds (3). Apparently streptolysin O is inhibited in an entirely different fashion.

The possibility was considered that phospholipid is combined with cholesterol in such a manner as to make it unavailable as an inhibitor of streptolysin O. This possibility prompted experiments employing a lecithinase to "liberate" cholesterol. Although most sera treated with lecithinase showed the expected increase in inhibition of streptolysin O hemolysis, the results were not consistent.

The action of *Cl. welchii* lecithinase upon human serum has been shown to affect profoundly the physical properties of serum beta-1 lipoprotein in studies employing ultracentrifugation and electrophoresis (29). The unstabilizing effect on serum lipids which is produced by the action of lecithinase (30) probably results in highly complex changes which cannot be interpreted simply as selective destruction of phospholipid. More likely such destruction results in the complete disruption of lipoprotein complexes. For example, when normal human serum which has been treated with *Cl. welchii* lecithinase is subjected to ultracentrifugation by the sedimentation-flotation method of Gofman and his associates (28), practically no beta-1 lipoprotein can be recovered. Also, the clear beta lipoprotein solution obtained from human serum by this method (28) becomes intensely milky when treated with lecithinase and loses its normal property of streptolysin S inhibition (31). The alterations in serum lipoproteins produced by the action of lecithinase is reflected variously by the inhibition of three different hemolysins: an increase in streptolysin O inhibition, no change in saponin inhibition, and a decrease in streptolysin S inhibition (31). The effect of *Cl. welchii* lecithinase upon rabbit sera could not be studied because this enzyme, for reasons not understood, does not react with animal sera (32).

The inhibition of streptolysin O hemolysis does not appear to depend entirely upon the ratio of cholesterol to phospholipid in serum. The enhancing effect of heparin upon the inhibition of streptolysin O by the sera of cholesterol-fed rabbits occurs in the absence of a significant change in this ratio. This effect of heparin may be related to the change in the size of lipoprotein molecules which occurs following injection of this substance (19, 33, 34). In addition, the diluted sera of rabbits who received Triton WR 1339 intravenously showed inhibition of streptolysin O hemolysis even though the cholesterol/phospholipid ratio of such sera was quite low.

It is apparent that the inhibition of streptolysin O hemolysis by serum lipids is a highly complex and as yet poorly understood phenomenon. The sensitivity of this hemolytic system to variations in the physicochemical state of serum lipoproteins does, however, afford an opportunity for empirical observations of the behavior of these lipoproteins in intact serum.

SUMMARY

Various types of hyperlipemic rabbit and human sera were tested as inhibitors of two hemolysins, streptolysin O and saponin, both of which are known to be inhibited by cholesterol. Certain types of sera showed distinctive properties as inhibitors of these hemolysins.

Streptolysin O hemolysis was strongly inhibited by the sera of rabbits fed cholesterol. This inhibition was enhanced by the administration of heparin to these animals. Inhibition of streptolysin O hemolysis appeared to be related to a high ratio of the concentration of serum cholesterol to serum phospholipids.

Human hypercholesterolemic sera did not inhibit streptolysin O except in rare instances where the ratio of serum cholesterol to phospholipid was unusually high. Enzymatic degradation of phospholipids by *Cl. welchii* lecithinase resulted, in most instances, in marked increase in the inhibition of streptolysin O hemolysis.

The inhibition of saponin hemolysis was quite different from that of streptolysin O. The inhibition of saponin appeared to be related in general to absolute cholesterol concentration rather than to cholesterol/phospholipid ratios.

The property of streptolysin O inhibition by sera appears to be closely related to the chemical structure and physical state of serum lipoproteins.

ACKNOWLEDGMENT

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THE PREPARATION OF PARTIALLY PURIFIED PLASMIN, AND AN ACCOUNT OF ITS INTRATHECAL USE IN PATIENTS

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The treatment of tuberculous meningitis is frequently hindered by the formation of exudate within the cerebrospinal spaces. This exudate may obstruct the cerebrospinal circulation and give rise to the complications of subtentorial, basal cistern, and spinal block. Furthermore, the presence of exudate predisposes to the occurrence of tuberculous arteritis, which is responsible for the cerebral and spinal cord softenings frequently found in this disease (1–4).

Cathie (5) introduced the use of intrathecal streptokinase to prevent the formation of, and to remove exudate. It was hoped that the use of this agent would reduce the incidence of complications, render chemotherapy more effective, and lessen the likelihood of relapse. Clinical trials in tuberculous meningitis with streptokinase have yielded contradictory results (6–9). The majority of investigators believe that its value remains unproven.

Streptokinase has no intrinsic proteolytic properties, but activates a precurser plasminogen to form a proteolytic enzyme plasmin, the action of which may be inhibited by body fluids (10). Streptokinase was used in the treatment of pleural infections by Tillett and Sherry (11), who showed that clinical response was dependent upon the demonstration of adequate fibrinolytic activity in these fluids. Investigators interested in intrathecal treatment have relied upon clinical response to assess the adequacy of the dose, and have for the most part employed a dose of 100 Christensen units (600 Cathie units) of streptokinase for intrathecal use.

The development of methods for the measurement of the various components of the plasminogen-plasmin system in biological fluids (12) led Fletcher (13) to determine the composition of the cerebrospinal fluid with respect to these components in normal persons, and in patients suffering from tuberculous meningitis. He showed that although the addition of streptokinase in sufficient quantity to cerebrospinal fluid in tuberculous meningitis would always produce fibrinolytic activity in the fluid, the concentration required was sometimes greater than could be administered to patients for fear of toxic reactions. Moreover, the administration of relatively large doses of streptokinase, shown to be adequate by *in vitro* testing, frequently failed to produce any free fibrinolytic activity *in vivo*. The evidence indicated that the irritant action of high concentrations of streptokinase on the meninges was sufficient to alter the composition of the cerebrospinal fluid, and to inhibit fibrinolytic activity. Fletcher concluded that the intrathecal administration of streptokinase was only of limited benefit in tuberculous meningitis, and that its use required close laboratory control.

Hamburger and Biehl (14) using rhesus monkeys have demonstrated that the irritant effects of intrathecal streptokinase produced a marked pleocytosis and a positive Pandy response in the cerebrospinal fluid, although the monkeys remained well.

It seemed that many, although not all, of the difficulties encountered with the clinical use of streptokinase were due to its role as an activator rendering its action entirely dependent upon the composition of the cerebrospinal fluid. The use of the enzyme itself, plasmin, rather than the activator, streptokinase, might be expected to offer certain advantages in therapy. The present communication describes the preparation of partially purified plasmin, and the clinical results following its use in tuberculous meningitis.

MATERIALS AND METHODS

A single batch of streptokinase-streptodornase ("Varidase" Lederle, No. 7-1089-121A) was used throughout. The relative proportion of streptokinase to streptodor-

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nase was high, each ampule containing 148,000 units of streptokinase and 17,000 units of streptodornase. The methods of measurement for the components of the plasminogen plasmin system have been described in a previous publication (12) and were recorded in Christensen units (15). Nitrogen determinations were made by a conventional micro-Kjeldahl method.

Preparation of plasminogen

All preparations were kept in the cold room at 4°C, whilst procedures with ether were carried out at 0°C. To insure complete precipitation, a period of 12 hours was allowed to elapse between each precipitation step, and subsequent centrifugation, which was performed in an International PR-1 angle head model at 0°C. Upon occasion, in the absence of ether, the temperature was allowed to rise to 20°C, to speed the solution of precipitates.

Blood (four to six weeks old) from the bank, preserved in glucose citrate mixture, was centrifuged in an Alfa-Laval model. Bottles showing obvious contamination or gross hemolysis were discarded.

Ether (100 ml.) was slowly added, with stirring, to 1 liter of plasma contained in an ice bath (16). The precipitate was discarded, and a further 150 ml. of ether was added to the supernatant, the mixture was placed in a bottle and frozen in contact with solid carbon dioxide (17). Twelve hours later it was thawed at 4°C, and centrifuged. The precipitate and the fatty ether layer were discarded.

The residue was left overnight in shallow dishes under a fan in the cold room to remove excess ether, and then dialyzed against running tap water for 12 hours. It was then diluted fivefold with distilled water, and the pH adjusted to 5.3 with 1 per cent acetic acid. The precipitate was taken up into 400 ml. of 0.225 M saline, and the pH adjusted to 7.4 with sodium hydroxide, after the addition of 20 ml. of 0.1 M phosphate buffer. This solution was filtered through a Whatman No. 52 paper, diluted tenfold with distilled water, and the pH adjusted to 5.3. The precipitate was dissolved in 300 ml. of 0.15 M saline. If the material was to be freeze-dried the pH was adjusted to 7.4, the final concentration of phosphate buffer being 0.005 M; if the material was to be sterilized the final pH was 6.7 and the concentration of phosphate buffer was 0.004 M.

Sterilization

Difficulty was encountered in sterilizing the various plasminogen preparations, since adsorption of material on to filter pads, and blocking of filters was frequently encountered. Filter aids (*e.g.*, celite) were not helpful. Material adjusted to pH 6.5 to 6.7 was centrifuged for one hour at 5,000 rpm in an angle head centrifuge, and then filtered through a Whatman No. 52 paper. The small Seitz filter (6 cm.) was prepared as follows: 200 ml. of 0.05 M phosphate buffer at pH 6.5, followed by 100 ml. of the preparation to be filtered were passed through the filter, and then discarded. Prepared in this manner the filter would now pass 600 to 800 ml. of preparation without blocking and without any significant loss of plasminogen.

Subsequent manipulations were performed in a germicidal ultraviolet chamber. Sufficient buffer to bring the pH to 7.4 was added. The preparation was assayed for plasminogen (it usually contained 400 to 800 units per ml. at this stage) and was diluted to bring it to 100 to 150 plasminogen units per ml. in 0.15 M saline buffered in 0.005 M phosphate buffer. The requisite amount of streptokinase was then added with the temperature at 4°C, and the material was stored at −20°C in screw capped bottles. The contents were stable for some months. Samples from each bottle, taken prior to storage, were planted into a tube of glucose broth and a tube of chopped meat medium, which were incubated at 37°C for 10 days, to test for sterility.

Purification ratio and recovery rate

The original serum averaged 100 units plasminogen per mg. N, whilst the processed material assayed at 5,000 to 10,000 units plasminogen per mg. N, representing a purification of 50 to 100 fold. The usual recovery rate of plasminogen was 25 per cent (range 15 per cent to 35 per cent). Equally important from the view of therapeutic use was the fact that inhibitor (expressed as equivalents μg trypsin per mg. N) had not been concentrated in the processed material (Serum inhibited 50 to 60 μg trypsin per mg. N, whilst this preparation inhibited 30 to 50 μg trypsin per mg. N).

Plasmin preparations for intrathecal use

Earlier work (12 and 13) had shown (a) that while streptokinase was an irritant to the spinal theca, when used in concentrations of 10 to 20 units per ml. cerebrospinal fluid this effect was relatively unimportant, (b) that where low concentrations of streptokinase were used on a solution of fixed plasminogen concentration, the plasmin activity produced was related to the logarithm of the streptokinase concentration, (c) that where the "optimal" concentration of streptokinase (that producing maximum fibrinolytic activity) was used, the plasmin activity was related to the cube root of the plasminogen concentration, and (d) that when the cerebrospinal fluid pathways were obstructed in tuberculous meningitis, the antiplasmin content of the cerebrospinal fluid might rise as high as 15 inhibitor units per ml.

These findings were interpreted as indicating that preparations of plasmin for intrathecal use should be activated with no more than 50 to 100 units streptokinase per ml., and should contain no more plasminogen than was required to produce an enzyme activity of 20 to 25 plasmin units per ml.

Plasminogen diluted to 100 to 150 units plasminogen per ml. and activated with 50 to 100 units streptokinase per ml., fulfilled these requirements. The activity of the various preparations used in this work ranged from 19 to 27 plasmin units per ml. Each ml. of solution then contained 0.02 to 0.01 mg. protein N per ml., and since the clinical dose of the material was found to range from

4 to 15 ml., a single therapeutic injection contained 0.04 to 0.3 mg. protein N.

The streptokinase used also contained streptodornase, and this resulted in the plasmin solution containing 5.5 to 11 units streptodornase per ml. This amount of streptodornase is many times less than that found in our experience to be therapeutically effective, and its presence is not further considered.

RESULTS

Plasmin solutions maintained at 37°C decay at a substantial rate. Figure 1 illustrates two decay curves typical of concentrations used therapeutically. Decay curves similar to those illustrated in Figure 1 were obtained when plasmin and cerebrospinal fluid were mixed and incubated at 37°C. These data suggested that the duration of effective fibrinolytic activity *in vivo* was unlikely to exceed 2 to 3 hours if concentrations of enzyme giving 2 to 5 units plasmin per ml. cerebrospinal fluid were used. Experimental confirmation of this point was obtained in six patients.

A further problem was posed by the fact that though one unit of plasmin per ml. will lyse 0.5 mg. of human fibrin at 37°C in 30 minutes if the fibrin is in intimate contact with the enzyme, fibrin deposited on the meninges will present a very much smaller surface for enzyme action. Crude experiments with glass models (13) suggested that the dose of plasmin should be increased 3 to 6 fold to compensate for this factor.

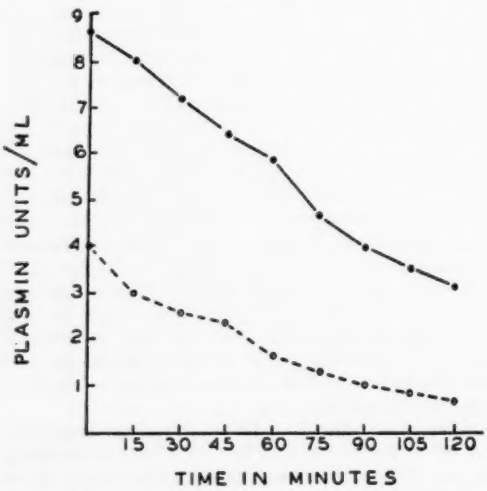

FIG. 1. DECAY OF PLASMIN ACTIVITY AT 37°C
● — ● solution containing 8.5 units per ml.
○ — ○ solution containing 4.0 units per ml.

Although the amount of fibrin deposited intrathecally cannot be measured, these considerations suggested that fairly high levels of plasmin would be required for successful intrathecal use. It was thought that one unit plasmin per ml. would probably represent a minimal level for successful therapy. In practice sufficient plasmin was injected to provide 2 to 5 plasmin units per ml. cerebrospinal fluid.

Estimation of the dose required for intrathecal treatment

The "plasmin" used contained plasmin, plasminogen, and streptokinase, whilst the cerebrospinal fluid of patients contained varying amounts of plasminogen, antiplasmin, and low concentrations of anti-streptokinase. The effect of mixing and dilution upon such systems is complex. Furthermore, the composition of the cerebrospinal fluid varies in different portions of the cerebrospinal spaces.

Figure 2 shows that the composition of the patient's cerebrospinal fluid would exert a considerable influence upon the level of fibrinolytic activity produced by the injection of plasmin. The dose suitable for each patient was ascertained by mixing plasmin with varying amounts of cerebrospinal fluid withdrawn from the patient, and after 15 minutes assaying for fibrinolytic activity. The dose was calculated by assuming that the injected enzyme would have mixed after 15 minutes, with 100 ml. of cerebrospinal fluid identical in composition with that previously withdrawn from the site of injection. Study of phenolsulphonephthalein dilution curves suggested that the error inherent in this assumption should not exceed ± 25 per cent. These estimates refer to patients with a free cerebrospinal circulation. Where a spinal block was present the volume of cerebrospinal fluid beneath the block was determined by a phenolsulphonephthalein dilution method, and calculations were based on this detemination. The levels of fibrinolytic activity calculated by these procedures are referred to as the predicted enzyme levels.

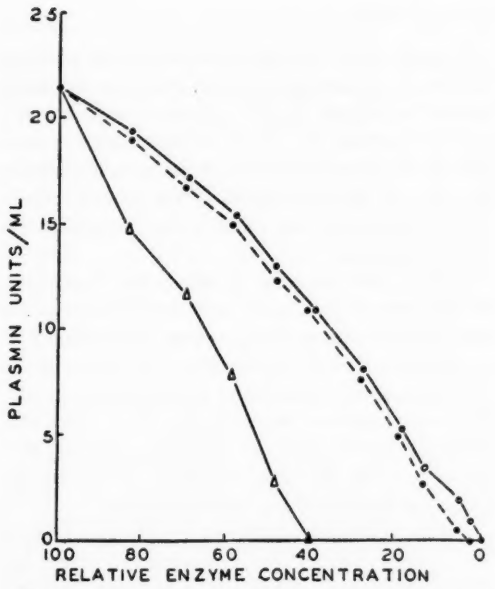

FIG. 2. ENZYME ACTIVITY FOUND WHEN PLASMIN IS DILUTED WITH CEREBROSPINAL FLUID TAKEN FROM PATIENTS SUFFERING FROM TUBERCULOUS MENINGITIS

○—○ Diluted with buffer.

●---● Diluted with cerebrospinal fluid of low inhibitor content (1.2 inhibitor units per ml. 12 plasminogen units per ml.) typical of ventricular cerebrospinal fluid.

△—△ Diluted with cerebrospinal fluid of high inhibitor content (15 inhibitor units per ml. 400 plasminogen units per ml.) typical of cerebrospinal fluid showing a Froin syndrome.

Mode of plasmin administration and method of sampling

Puncture of the subarachnoid space was made in the desired location, and the dose of plasmin, usually 4 to 8 ml., but on occasion as much as 15 ml., was diluted with cerebrospinal fluid in the syringe. The mixture was slowly injected. The syringe remained attached to the needle, and a further two withdrawals and reinjections were made to promote mixing. The needle remained in the subarachnoid space, and 15 minutes later, 5 ml. of cerebrospinal fluid was withdrawn for analysis using a fresh syringe.

The time of 15 minutes for sample withdrawal was selected firstly because previous work had shown that plasmin levels were usually highest at this time (13), and secondly because most patients became worried if a needle was left in the subarachnoid space for longer. Moreover, three experiments involving simultaneous ventricular and cisternal sampling had shown that at least with respect to these compartments the difference of plasmin concentrations at the time, was less than 30 per cent.

Correlation of predicted enzyme values and those obtained by sampling

Figure 3 shows the predicted and actual plasmin levels obtained by sampling after injection of plasmin in 18 patients suffering from tuberculous meningitis and with a free cerebrospinal circulation. There is a reasonable correlation between rise of enzyme concentration predicted from *in vitro* tests, and rise of enzyme activity found on sampling. The main interest of these figures is to show that consistent and high levels of fibrinolytic activity can be produced in the spinal theca. These findings differ markedly from those with streptokinase, where *in vivo* levels were under similar circumstances extremely low or altogether absent.

Figure 4 shows the predicted and actual plasmin levels when plasmin was injected below a spinal block. Despite the high level of inhibitor invariably present, reasonable levels of fibrinolytic

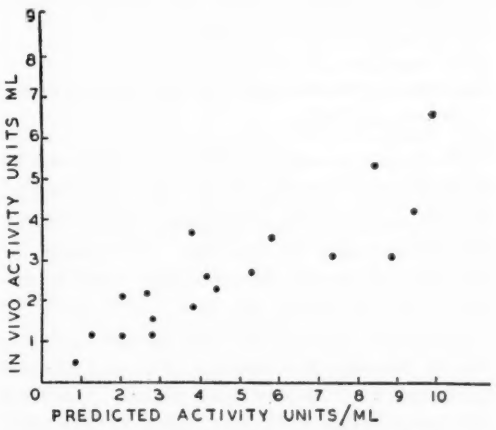

FIG. 3. COMPARISON OF PREDICTED ENZYME ACTIVITY (ABSCISSA) AND THE ACTUAL ENZYME ACTIVITY OBTAINED BY SAMPLING (ORDINATE)

Cases with unobstructed cerebrospinal circulation (18 observations).

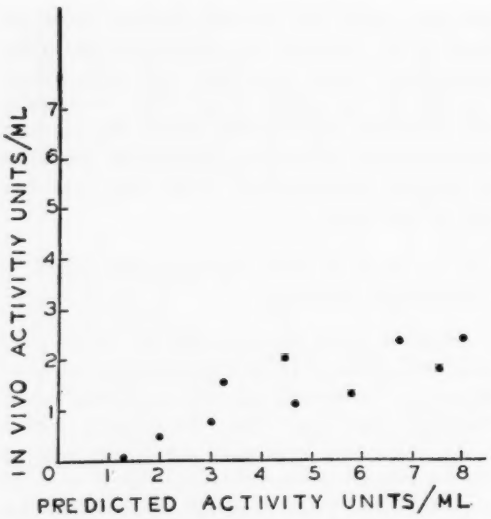

FIG. 4. COMPARISON OF PREDICTED ENZYME ACTIVITY (ABSCISSA) AND THE ACTUAL ENZYME ACTIVITY OBTAINED BY SAMPLING (ORDINATE)

Cases with spinal block, the enzyme being injected below the block (10 observations).

activity were produced below the block. However, the figures suggest that with the present preparations, high levels of fibrinolytic activity will not be obtained. These figures are in contrast to those obtained with streptokinase, where in all cases under such circumstances there was failure to demonstrate any fibrinolytic activity.

The effect of plasmin upon the composition of the cerebrospinal fluid

The degree of meningeal irritation produced by the intrathecal injection of plasmin was assessed by examination of the cerebrospinal fluid prior to, and 24 hours after an injection. Thirty-three injections (100 to 400 units plasmin) were so followed, and the results are shown in Table I.

This table demonstrates that though the injection of plasmin on occasion results in a marked pleocytosis and an increased protein content of the cerebrospinal fluid, the degree of meningeal irritation produced, as judged in this manner, is not excessive. The figures for chloride and glucose concentrations were substantially unchanged, and have not been tabulated.

Clinical toxicity

A total of 83 intrathecal injections of plasmin has been administered to 15 patients, the least number of injections per patient was 3, and the greatest number 11. Many of these patients were gravely ill (some in coma) at the time of plasmin therapy, so that assessment of the patient's reaction to treatment had to be made on rather uncertain grounds.

Undesirable reactions to treatment have been divided into severe, mild, and not detected. An unexplained rise of temperature exceeding 2°F or transient clinical deterioration was taken as indicating a severe reaction in unconscious patients. The same criteria were employed in conscious patients; severe headache, copious vomiting, dizziness or other disabling symptoms were also regarded as indicative of a severe reaction.

Severe reactions numbered 14 (17 per cent of injections). Case 1 died following a cisternal injection of plasmin, though it is probable that her death was coincidental. Case 3 apparently became sensitized to the streptokinase component, and developed a marked pyrexial response to later injections. Case 5 developed a convulsion during one injection. Case 6 developed a temporary spinal fluid block. Case 7 experienced an immediate reaction with mental confusion, tachycardia, and a flushed skin. These case histories are given later.

Any unusual symptom or exaggeration of any symptom previously present was considered a mild reaction. These numbered 20 (24 per cent of injections).

No detectable adverse reaction occurred after 49 injections (59 per cent of injections).

The incidence of adverse clinical reaction to

TABLE I

The effect of intrathecal plasmin upon the patients' cerebrospinal fluid (33 observations in patients suffering from tuberculous meningitis, dose 100 to 400 units, second examination 24 hours after the injection)

Number of Observations
(Change from Patients' Pre-Treatment Level)

Nature of change	Lowered below −20%	Unchanged ±20%	Raised +20% to +100%	Raised over +100%
Cells	0	12	12	9
Protein	6	19	5	3

plasmin appeared to be high, but any untoward symptom was regarded as a reaction. It is possible that a number of the complaints that followed the administration of plasmin were not related to the toxic properties of the preparation itself, but to lysis of intrathecal exudate; indeed some of the reactions to plasmin resembled those seen following the intrathecal injection of tuberculin (18).

No case of clinical jaundice was detected in this series, but further evidence as to the safety or danger of these preparations with regard to the transference of homologous serum jaundice is not available.

Illustrative case histories

Tubercle bacilli were isolated from the cerebrospinal fluid of all patients described in this section. In all cases the organisms were sensitive to streptomycin. In cases 3, 4, 5 and 6 the organisms were shown to be sensitive to isoniazid. All patients were treated with intramuscular and intrathecal streptomycin; cases 1, 2 and 7 received in addition 16 Gm. p-aminosalycilic acid daily; cases 4, 5 and 6 received either 10 mg. per Kg. or 200 mg. daily of isoniazid; whilst case 3 received both p-aminosalycilic acid and isoniazid. These case histories were selected to include all patients with severe reactions to plasmin, and also cases with histories where the suggestion of benefit to the patient from plasmin therapy appeared strong. Fifteen patients received plasmin treatment because of some degree of unsatisfactory response to conventional treatment. If case 1 is included, there were two deaths, whilst one patient (case 5) is disabled.

Case 1 (female, age 47), suffering from pulmonary tuberculosis and tuberculous meningitis, had responded poorly to a six months' course of treatment. A ventriculogram had revealed grossly dilated ventricles and an absence of subarachnoid air over the cortex. She was in deep coma, had developed pulmonary edema, and appeared to be in extremis. The first preparations of plasmin had just been made, and she was given a cisternal injection (100 units). Death occurred 30 minutes later.

Post mortem examination revealed extensive pulmonary tuberculosis, advanced signs of chronic tuberculous meningitis with a subtentorial block, and gross hydrocephalus. There was no indication of a misplaced cisternal injection. It is probable that her death was coincidental, especially as the same batch of material was later successfully used in other patients.

Case 2 (female, age 15). Admitted in February, 1952 when she was presented as an ill girl with a high fever, mental confusion, head retraction and a marked Kernig sign. Both fundi showed early papilledema with numerous choroidal tubercles. Coarse rales were heard throughout the chest and the chest X-ray revealed gross miliary shadowing.

By mid-April her condition had improved, but at this time she developed a spinal block. Two injections of plasmin (300 units) were given below the block, after which the block resolved. This relief was only temporary, and ten days later the cisternal pressure rose to 300 mm.+, the spinal block reappeared, she lapsed into deep coma and gross papilledema in both fundi. A ventriculogram revealed generalized ventricular dilatation and an absence of air over the cortex. Simultaneous ventricular and cisternal puncture revealed that the ventricles communicated freely with the cisterna magna. The diagnosis of subtentorial block appeared to be certain.

Four injections of plasmin (200 units each) during the next eight days *via* the ventricular route produced a dramatic change in her clinical condition. The gross papilledema disappeared, she recovered from coma, and became rational, mentally alert, and cheerful. There was a gradual disappearance of pyrexia, though the spinal fluid block persisted.

Three weeks after the last plasmin injection her temperature suddenly rose to 104, she developed tachycardia of 200, and died 24 hours later.

Post mortem examination revealed extensive miliary tuberculosis, which had apparently responded little to treatment. Numerous vascular lesions due to tuberculous arteritis were found in the brain stem and upper cervical cord, and these lesions were probably the cause of her death. Careful examination of the tentorial opening showed that although it was partly occluded by exudate, there was adequate and free communication above and below the membrane. The ventricular system was of normal dimensions.

Case 3 (female, age 30). Admitted in August, 1952 when her clinical condition was grave; she was drowsy, irrational and showed marked signs of meningeal irritation. A left external rectus palsy was present, but the fundi were normal. A chest X-ray revealed the classical "snow storm" appearance of gross miliary tuberculosis and numerous scattered coarse rales were audible on physical examination.

Her response to treatment was slow, but at the end of one month she was rational, the external rectus palsy had disappeared, and rales were no longer heard over the chest. She continued to improve during the next three weeks, but at the end of September, 1952 she developed a spinal fluid block. Early in October she developed papilledema, and became comatose, whilst the cisternal pressure, which had previously averaged 150 mm., suddenly rose, and consistently exceeded 300 mm.

Burr holes were drilled, and one week later during which time her condition had further deteriorated and

she had developed gross papilledema, a ventriculogram was performed. The ventriculogram revealed some degree of generalized ventricular dilatation, and an absence of air over the cortex. Simultaneous cisternal and ventricular punctures demonstrated free communication between the ventricles and the cisterna magna, which confirmed the diagnosis of subtentorial block.

She was treated with eight injections of plasmin over the next three weeks; 200 to 300 units were given upon each occasion by the ventricular route. Following the second injection and subsequently there was rapid clinical improvement. At the end of the course she was fully conscious and rational, and the ventricular pressure averaged 150 mm. A ventriculogram demonstrated the ventricles to be of normal size, whilst air was seen over the cortex. The papilledema had greatly decreased, though the disc margins were still abnormal.

During November, 1952 three injections of plasmin were given by the lumbar route with the intention of resolving the spinal fluid block. Each injection was followed by increasing pyrexia and systemic upset. It was thought that the initial course of injections had sensitized her to streptokinase, a conclusion supported by the fact that she tolerated an intrathecal injection of plasminogen without disturbance. She was discharged from the hospital in April 1953, and has since remained well, though her spinal fluid block has persisted.

Case 4 (female, age 30). Admitted in April, 1953. Her general condition was good. Examination revealed a left external rectus palsy and signs of meningeal irritation. The chest X-ray showed small, apparently inactive adult type tuberculous foci at both apices.

A week after admission she developed a left superior oblique palsy, and papilledema appeared. The cerebrospinal fluid pressure remained low, but she became irrational and drowsy, and began to vomit frequently. A tentative diagnosis of basal cistern block was made.

Six weeks after admission all signs had progressed and she was gravely ill and had lost much weight. Her cerebrospinal fluid pressure was still low, and the diagnosis of basal cistern block was now felt to be very likely. Plasmin (100 units) was administered by the cisternal route, shortly after which she complained of severe pain in the neck and vomited copiously. The next day the superior oblique palsy had disappeared.

Two days later 200 units of plasmin were given by the cisternal route without upset. Three days later 200 units were administered. This injection was followed one hour later by marked dizziness, copious vomiting and a temperature of 102°F. She recovered in about eight hours and 24 hours later the left external rectus palsy had disappeared. Twelve days after the start of plasmin therapy her general condition was much improved, she was rational, eating well, vomiting had ceased, and the papilledema had practically disappeared, though she still complained of diplopia upon looking to the left. Her cisternal fluid protein dropped from a level of 300 mg. per cent to 150 mg. per cent during the period of therapy.

Her clinical progress has since been excellent, she has recovered her pre-treatment weight, and binocular vision has slowly been achieved. She was discharged from hospital during November, 1953.

Case 5 (female, age 35). This patient, a gravely ill woman, suffered also from advanced renal and vesical tuberculosis, when she was admitted during December, 1952.

One week after admission papilledema appeared followed by paralysis of the left lateral rectus and left inferior oblique muscles. Despite a low cerebrospinal fluid pressure, the papilledema increased rapidly, becoming gross at the end of four weeks. At this time the cerebrospinal fluid pressure suddenly rose to over 300 mm., the patient's condition worsened, and she began to vomit frequently. The diagnosis of basal cistern block complicated by a recent subtentorial block was thought to be likely, but owing to technical difficulties, satisfactory air injection studies were not completed. However, test of phenolsulphophenolthalein excretion from the ventricles was decreased, only 23 per cent of the total dose being excreted in two hours.

On the thirteenth of January, 1953, she was given 400 units of plasmin by the lumbar route without incident, and on the next day she seemed better. On the sixteenth of January, 400 units of plasmin were given by the ventricular route. During the injection she experienced a transient convulsion, the pupils became dilated and the right arm twitched. She recovered after three minutes but vomited later. On January nineteenth, the inferior oblique palsy had disappeared, and an additional 200 units of plasmin were given by the lumbar route without incident. On the twenty-sixth her general condition was greatly improved, and the papilledema had regressed to a marked degree. By the twenty-third of February the optic fundi were nearly normal, the cerebrospinal fluid pressure was 200 mm., and the left rectus palsy had disappeared so that binocular vision was possible. By mid-February she was rational, and the urinary excretion rate of phenolsulphophenolthalein injected into the ventricles had returned to normal.

In April symptoms and signs of optic atrophy were found, and by the end of June visual acuity was reduced to finger counting at one yard. Since this time her vision has improved, but the loss of visual acuity is still severe.

By July her cerebrospinal fluid was within normal limits and has remained so. The renal and vesical tuberculosis, however, progressed and she has since had her ureters transplanted into a blind ileostomy because of a contracted bladder.

Case 6 (female, age 18). Admitted during February, 1953. Her general clinical condition was good, though early papilledema was present.

In April the papilledema, which had been slowly increasing in degree, had become gross, and her condition commenced to deteriorate, although the cerebrospinal fluid pressure remained low. A diagnosis of basal cistern block appeared probable. She had also complained of severe pain in the legs, which had been attributed to involvement of the posterior roots by exudate.

Two hundred and twenty units of plasmin were given

by the lumbar route without discomfort. Four hours later she complained of severe aching in the chest, examination showed her to be tender over the thoracic spines, and light percussion over these spines intensified the chest pain. She was given analgesics and the pain disappeared.

Three days later the papilledema had decreased, and her general condition had improved. An injection of 110 units of plasmin was given by the lumbar route. Symptoms similar to those experienced earlier developed after three hours, but this time the intensity of the pain was greater. The next day lumbar puncture revealed a Froin syndrome in the cerebrospinal fluid and a negative Queckenstett response, but 24 hours later these anomalies were absent, and the lumbar cerebrospinal fluid protein was 600 mg. per cent, which was the same as before plasmin therapy.

One further injection of 150 units plasmin was given, but upon this occasion the injection was tolerated well. During the fortnight's treatment with plasmin the papilledema decreased greatly (from 4 plus to 1 plus). The pains in the legs were entirely relieved following the third plasmin injection. Her clinical condition was greatly improved, and her subsequent progress was satisfactory. There was no impairment of visual acuity. She was discharged from hospital in October, 1953.

Case 7 (female, age 23). Three months' treatment had produced a substantial improvement in this patient's condition. She had complained, for three weeks, of severe pains in the legs, for which no cause could be found, and which was controlled only by large doses of analgesics. It was thought that the lumbar sensory roots were surrounded by exudate.

Two minutes after a lumbar injection of 200 units of plasmin, she became dizzy, shouted that she was dying, sweated profusely, developed a flushed skin, and her pulse rate rose from 70 to 160. An injection of sodium phenobarbitone produced rapid relief, and 30 minutes later she was apparently well.

This particular batch of material had been buffered with 0.005 M citrate buffer. The use of citrate buffer was stopped, since temporary depression of the ionized calcium in the cerebrospinal fluid could have been responsible for her symptoms. This patient later tolerated two injections of plasmin buffered with 0.005 M phosphate without complaint. The pains in the legs disappeared after the second injection. She has since been discharged from hospital and remains well.

DISCUSSION

The findings reported here show that high levels of fibrinolytic activity can be safely produced intrathecally by the injection of plasmin. Further, it is possible to produce reasonable levels of fibrinolytic activity with plasmin even when the cerebrospinal fluid shows a Froin syndrome, a condition previously shown to be a bar to the production of fibrinolytic activity by injections of streptokinase (13).

The purpose of the clinical investigation was to determine whether plasmin would lyse tuberculous exudate *in vivo*. Subtentorial and basal cistern blocks were selected as suitable test lesions since both are known to be caused by exudate formation, the presence or absence of either complication can be diagnosed during life, and the usual outcome of the untreated lesion is unfavorable.

The case histories indicate that the levels of plasmin, which can be attained by intrathecal injection, are capable of lysing exudate *in vivo*. Cases 2 and 3 showed regression of hydrocephalus under treatment, an extremely rare occurrence. Lorber (19), who cites the literature fully, believes that hydrocephalus either remains stationary or progresses, despite the clinical improvement that sometimes occurs. He observed only one patient in whom there was evidence that the degree of hydrocephalus regressed. The diagnosis of basal cistern block during life is difficult, but the evidence points strongly toward this diagnosis in cases 4, 5 and 6. All made an apparently striking improvement on plasmin, though case 5 eventually developed optic atrophy.

The treatment of spinal fluid block was regarded as a secondary aim, since initially, the dangers of this condition to the patient were not fully appreciated. Poor results were obtained when treating spinal fluid block with plasmin. Ten attempts (*i.e.*, single injections) to lyse spinal blocks in three patients were made with two temporary successes. The reasons for this difficulty are readily appreciated from post mortem study, since the surface available for enzyme action is extremely small, compared to the total volume of the block.

Plasmin having been shown to be effective *in vivo*, the basis and timing of treatment required examination. Subtentorial and basal cistern block are most dangerous lesions causing death, intellectual deterioration, blindness, and other sequelae. It is clearly unjustifiable to allow patients to undergo the hazards inseparable from the developed lesion, if prophylactic treatment is feasible. Brooks, Fletcher, and Wilson (2) have recently shown that exudate formation around the spinal cord leading to tuberculous arteritis is responsible for the appearance of transverse myelitis found in

this disease. They showed that, where exudate formation was gross, the frequency and severity of the lesions were notably increased. Here, too, prophylactic treatment is indicated, particularly in view of the indifferent results obtained by the treatment of established spinal block.

It is believed (20) that the use of isoniazid has greatly improved results in the treatment of tuberculous meningitis, and our experience supports this view. However, the need for some means for removal of exudate is underlined by the fact that four patients in this series and three patients cited by Brooks, Fletcher, and Wilson (2) were treated with streptomycin and a dose of isoniazid that gives satisfactory intrathecal levels (21).

Prophylactic treatment would have the further advantage that small doses of plasmin (100 to 200 units) would be adequate, and that the larger doses of 400 or more units would no longer be necessary. Fibrinolytic enzymes cannot lyse organized tissue, and thus in every patient the earlier that treatment can be started, the better should be the end results. Further, prophylactic treatment should lead to more favorable results than treatment of the established lesion, since the surface available, for enzyme action, and the access of the enzyme to the surface will both greatly diminish once the cerebrospinal fluid circulation is partially occluded.

The preparations of plasminogen used in this study were of a comparatively crude nature. Christensen and Smith (22), by means of their acid extraction process, have prepared material 5 to 10 times as pure, and Kline (23) by a modification of the Christensen process claims even greater purity. Unfortunately, material prepared by acid extraction has so far proved almost impossible to sterilize, owing to its physical state of dispersion. It is probable that much of the difficulty in completely activating solutions of plasminogen is due to the impurity of the streptokinase available, and further improvements in the preparation of this material would be helpful. The recent review of Christensen (24) provides an able discussion of the controversy concerning the mechanism of plasminogen activation.

The present preparations, despite their imperfections, would appear to fulfill the essential needs for clinical work. They were developed with the purpose of treating tuberculous meningitis, but it is clear that their use could be extended to the treatment of other forms of meningitis, where exudate formation is troublesome. Indeed, plasmin would appear to have a wide field of usefulness in a variety of pathological states, when, for one reason or another, *in vivo* streptokinase activation is not feasible.

SUMMARY

1. A method for the preparation of partially purified plasminogen is described. The preparations contained 5,000 to 10,000 units plasminogen per mg. N. This material after sterilization and activation with streptokinase was suitable for intrathecal use.

2. Eighty-three injections of plasmin were administered to 15 patients suffering from tuberculous meningitis. The incidence of systemic reaction was apparently high, but the degree of meningeal reaction, as judged by changes in the composition of the cerebrospinal fluid, was not great.

3. The injection of plasmin gave rise to high levels of intrathecal fibrinolytic activity in patients suffering from tuberculous meningitis. This finding contrasted sharply with the results obtained following the injection of streptokinase.

4. Patients suffering from subtentorial and basal cistern block were treated with plasmin to determine whether tuberculous exudate would undergo lysis *in vivo*. The results indicated that exudate had undergone lysis *in vivo*.

5. The clinical results obtained suggested that the use of plasmin as a therapeutic agent should be further investigated.

ACKNOWLEDGMENTS

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DISTRIBUTION AND DEGRADATION STUDIES WITH INSULIN-I^{131} †

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Diabetes mellitus is of obscure etiology, often presenting profound metabolic derangement with a sparsity of morphopathologic change. Insulin reverses many of the metabolic changes, yet its mechanism of action is poorly understood; furthermore, in most patients with diabetes mellitus, the fundamental causes of the insulin insufficiency are not known. An important approach to this challenging problem is the study of insulin metabolism. Such a study has been hampered by unsatisfactory assay techniques, and, as a result, very little is known even of the fate of insulin. The use of labeled hormone greatly facilitates such a study, and, in this report, the validity of such an approach is considered, and distribution and degradation studies using I^{131}-labeled insulin are described.

METHODS AND MATERIALS

Insulin-I^{131}

Crystalline zinc insulin [3] was labeled with I^{131}, either by the method of Ferrebee, Johnson, Mithoefer, and Gardella (1), or by a method of the Abbott laboratories. Specific activity ranged from 4 to 1008 μc per mg. The radioactivity of the insulin-I^{131} stayed with the protein fraction in trichloracetic acid precipitation, in dialysis, and in chromatography.[4] It had the same mobility as unlabeled insulin in paper electrophoresis (2).

The potencies of three different lots of insulin-I^{131}, two prepared by the Ferrebee technique, and one by the Abbott method, were tested in the acceleration of the glucose uptake of rat diaphragm *in vitro*. Each determination measured the glucose uptake of eight quarter diaphragms as described by Groen, Kamminga, Willebrands, and Blickman (3). Samples and controls were run in duplicate. One dose level in the sensitive part of the dose response curve was used. In all instances the insulin-I^{131} gave the predicted response, and no loss of activity was demonstrated.

In a control experiment, a sample of insulin, 27 units per mg., was treated with I^{127} instead of I^{131}. In all other respects, the insulin was iodinated exactly as in the Abbott insulin-I^{131} method. An *in vivo* bioassay was performed on this sample by the Lilly Research Laboratories. A standard mouse convulsion test was used initially to determine the potency of the insulin. Three assays were run, using 72 mice each time. The mouse assay was then checked on rabbits using a crossover technique. No definite loss of activity was demonstrated.[5] Further bioassays, using radioactive material, are planned.

Experimental animals

Unless otherwise noted, all animal experiments were performed as follows. Male Sprague-Dawley rats were used. They were kept at 80° F., and fed a standard diet of Purina Fox Chow with vitamin supplements. To ensure standard conditions of carbohydrate metabolism, food and water were replaced by 10 per cent dextrose in 0.9 per cent saline (10 per cent D/S) 17 hours before each experiment. This solution was removed 2 hours before the experiment, and the animals fasted thereafter. The isotopic preparation (10 μc or less), was injected into the tail vein in 0.5 ml. of 0.9 per cent saline over a period of 15 seconds, with the animal under ether anesthesia. The doses were constant in any one experiment, and in all rat experiments were within the range, 0.2 to 0.8 units. Fifteen minutes later, again under anesthesia, the rat was exsanguinated by puncture of the dorsal aorta, the tissues were removed, aliquots weighed on a Roller-Smith balance, and their radioactivity determined.

Measurement of radioactivity

Since the radioactivity of insulin-I^{131} itself was at least 95 per cent precipitable with trichloracetic acid (TCA), it was assumed that non-precipitable radioactivity repre-

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[3] This preparation was kindly supplied by Eli Lilly and Co. It contained 26 or 27 units per mg., and only minimal amounts of hyperglycemic factor.

[4] Ascending paper partition chromatography was performed using the butanol phase of a system composed of n-butanol: glacial acetic acid: water, (78: 5: 17).

[5] In a previous publication (4), this bioassay was reported as showing some reduction in the biological activity of the labeled insulin, but subsequent information demonstrated that this was an error due to a misunderstanding regarding the amount of starting material.

sented breakdown products of the insulin-I^{131} molecule. Accordingly, the radioactivity in all tissue and liquid samples was divided into two fractions, the TCA-precipitable, and the TCA supernatant fractions. In this procedure, the tissues were promptly minced and homogenized in 10 per cent TCA, and blood stirred in 20 per cent TCA. The supernatant was poured off after centrifugation, and the precipitate was washed once with 10 per cent TCA and again centrifuged. The supernatant fractions were pooled and diluted to 30 ml. with water. A second wash was found to remove less than 3 per cent of the radioactivity remaining in the precipitated fraction, and was therefore not used. The precipitated fraction, hereinafter noted as insulin *, was made up to 30 ml. with 30 per cent potassium hydroxide. Each specimen was then assayed for radioactivity in a well-type gamma counter.

The concentration of radioactivity in each tissue and fluid was calculated as the per cent of the dose per gram or ml. This figure was multiplied by the $\frac{\text{body weight}}{100}$, a dilution factor, to allow comparison of data on animals of different size, and, in short time intervals before appreciable excretion had occurred, to afford comparison of the concentration in a specific tissue or fluid with that in the body as a whole. This ratio was referred to as $\frac{[T]}{[B]}$. Any value above 1 indicated that the isotope was more concentrated in the respective specimen than it was in the body as a whole.

Tissue for radioautography was obtained from an adult male rat 15 minutes after the intravenous injection of 200 μc of insulin-I^{131}. The tissue was fixed in Bouin's solution, imbedded in paraffin, and 10 μc sections were floated on Kodak lantern slide medium plates for 24-hour exposure. They were then developed in the conventional manner, and stained with hematoxylin and eosin.

Distribution in humans

Insulin-I^{131} was administered to agonal patients in doses of 2.9 to 4.2 units, containing 30 to 40 μc of radioactivity. It was injected intravenously within one minute, and glucose solution was infused constantly thereafter. Aliquots of tissue were obtained at autopsy, weighed, and their total radioactivity determined in the well-type gamma counter. The results were expressed as per cent dose per gram $\times 10^{-3}$, without correction for body weight.

EXPERIMENTAL PROCEDURES AND RESULTS

Distribution in rats

The concentrations of radioactivity in rat tissues 15 minutes after intravenous insulin-I^{131} are shown in Figure 1. Each bar represents the average concentration in that tissue from a group of at least 5 rats, weighing between 180 and 264 Gm. The gastrocnemius was used as a representative muscle, and tibia as a representative bone. Fat was obtained from the perirenal area. Urine was collected in one group of rats 15 minutes after the administration of insulin-I^{131} by tying off the urethra and washing out the bladder with saline.

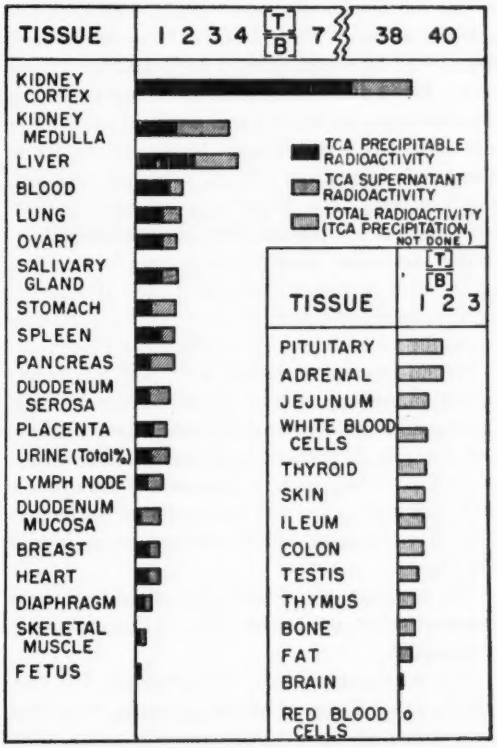

FIG. 1. CONCENTRATION OF RADIOACTIVITY IN RAT TISSUES 15 MINUTES AFTER I.V. INSULIN-I^{131}

Concentration, $\frac{[T]}{[B]}$, was calculated by multiplying the per cent of the dose of I^{131} per gram of tissue, by the $\frac{\text{body weight}}{100}$, so that values greater than 1 represent concentrations of radioactivity greater than that in the body as a whole. Urine was not weighed, so is recorded as per cent of the dose.

The two components of the total radioactivity, the trichloracetic acid (TCA) precipitate and the TCA supernatant, were determined for most tissues. Insulin-I^{131} is TCA precipitable, and supernatant radioactivity therefore consisted of breakdown products of the insulin-I^{131} molecule. Total radioactivity only, was determined on the tissues in the insert.

Note that the renal cortical concentration was so high that the scale had to be interrupted. The ratio of TCA precipitate to supernatant is accurately represented however.

The red blood cells were washed 4 times in 0.9 per cent saline. White blood cells were obtained from blood by centrifuging a 1 : 5, dextran (1 per cent) : blood dilution for 5 minutes, and aspirating the material over the red cell layer. The aspirate was diluted 1 : 4 with Hanks' buffer and the white blood cells centrifuged down, removed, weighed, and counted. The blood was originally drawn from the rat 10 minutes after the insulin-I^{131} injection, since the white cells could not be removed for at least 5 minutes thereafter, and it was desired to have a measure of concentration comparable to the 15 minute study in the other tissues.

Insulin-I^{131} concentration in thyroid was determined on a group of rats which received 100 mg. of propylthiouracil by stomach tube 4 hours before, and 100 mg. of potassium thiocyanate subcutaneously 1 hour before the experiment. This procedure was used to prevent accumulation in the thyroid of radioactive iodide released from insulin (2, 4).

The contents of the gastro-intestinal tract were removed before the radioactivity of this organ was determined.

The ovaries were from non-pregnant, 193 Gm. female rats. Data on breast, placenta, and fetus were from a group of 4 pregnant female rats, averaging 310 Gm. in weight.

This distribution study showed (Figure 1) a marked concentration of radioactivity in the renal cortex, over 9 times greater than that in any other tissue. This finding was further investigated in studies reported in a later section of this paper.

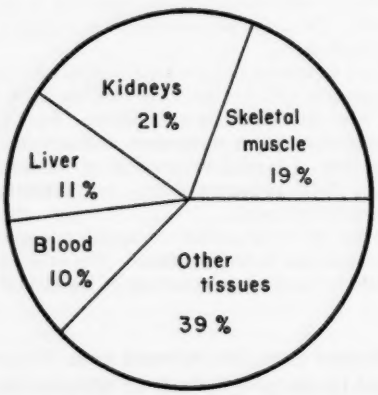

FIG. 2. TOTAL RADIOACTIVITY IN ENTIRE RAT TISSUES 15 MINUTES AFTER I.V. INSULIN-I^{131}

Liver had approximately twice the concentration of the blood. The concentration in the brain was very low and little was in the urine. Concentrations were not marked in the gastro-intestinal tract.[6] Radioactivity was found in white blood cells, but not in red blood cells. A higher concentration was found in ovary than in testis. The main tissues with concentrations less than the body as a whole, were bone, fat, and muscle. Heart and diaphragm had greater concentrations than skeletal muscle, the latter being relatively low. When total muscle mass was considered however,[7] and the total radioactivity in liver, kidney, muscle, and blood was plotted, as in Figure 2, muscle was seen to be a major site of localization.

Time studies

In the tissues of principal interest—liver, kidney, muscle, and blood—the concentration of radioactivity was determined at varying times after insulin-I^{131} injection. Three or more rats were used in each time period. Their average weight was 195 Gm. Those sacrificed at 2, 5, and 15 minutes were prepared in the usual manner. Those sacrificed at 1, 2, 4, and 6 hours received 10 per cent D/S for 17 hours up to the time of the insulin-I^{131} injection, and then were fasted. The insulin-I^{131} was given intravenously and the animals sacrificed after the appropriate time had elapsed. To ascertain the rate of breakdown of the labeled hormone, trichloracetic acid (TCA) precipitation was used, and the precipitable and supernatant tissue and blood radioactivity separated as described above. Supernatant radioactivity was considered to consist of breakdown products of insulin-I^{131}.

The time curves are shown in Figure 3. Insulin * (TCA precipitable radioactivity) concentration reached a peak in liver, kidney, and muscle, in from 5 to 15 minutes, and then fell rapidly, paralleling that in blood. After 1 hour, the fall in insulin * concentration was slight. Supernatant radioactivity appeared within the first few minutes after the insulin-I^{131} injection and increased in concentration in the tissues and blood in the

[6] Preliminary observations (2, 4) of high concentrations of radioactivity in the stomach may have been due to the presence of free I^{131} in early insulin-I^{131} preparations.

[7] The blood volume of a 190 Gm. rat was estimated as 10 ml., and total muscle mass as 85 Gm.

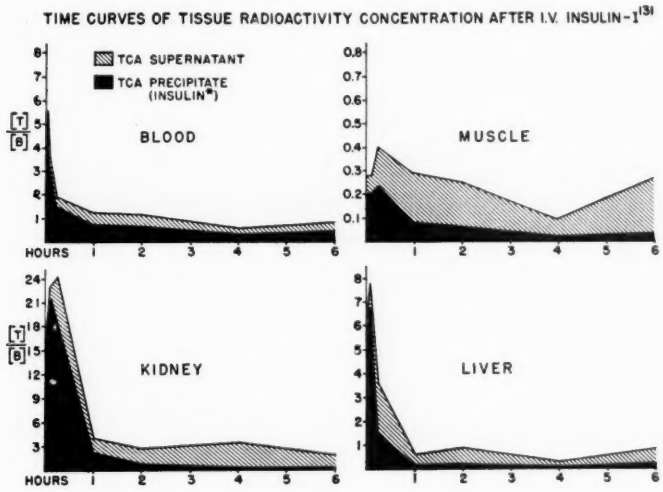

FIGURE 3

Note the rapid increase in concentration of supernatant radioactivity. Insulin-I^{131} itself was essentially completely precipitable by trichloracetic acid (TCA). Supernatant radioactivity therefore represents insulin-I^{131} degradation.

first 15 minutes. At later times, the concentrations of supernatant radioactivity varied without any consistent trend, and appreciable quantities of radioactivity were excreted in the urine in a non-precipitable form.

Excretion studies

Sixteen rats, averaging 211 Gm. in weight, were used. After the intravenous injection of insulin-I^{131}, from one to three rats were placed in each metabolic cage for the collection of urine and feces at various times. Two rats, in restraining cages, had total bile collections through polyethylene bile duct cannulae. For collection periods up to 6 hours in length the rats were given only 10 per cent D/S preceding, and nothing during, the experiment. When collections were continued for 24 or 48 hours, the rats were fed *ad libitum*. Feces were homogenized in the Waring blendor. All specimens were precipitated in 10 to 20 per cent TCA with dried human plasma added as carrier protein.

The results are shown in Figure 4. Each bar represents the average excretion of from two to four animals. The percentage excreted in the urine was many fold that in bile and stool. It is evident that the large part of the I^{131} was not precipitated by TCA. At the end of 6 hours, only 0.44 per cent of the dose was present in the urine in a protein precipitable form; even less was in the bile. At 24 hours only 1.7 per cent could be precipitated from the urine, and 0.8 per cent from

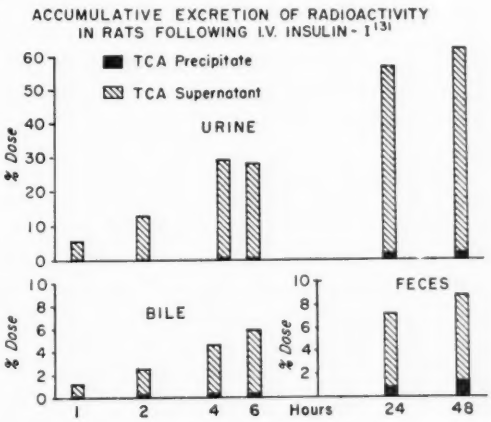

FIGURE 4

Bile and feces are plotted on the same scale, but urine is plotted on a smaller scale. Time is plotted logarithmically. Very little of the excreted radioactivity retained the property of insulin-I^{131} of precipitation by TCA.

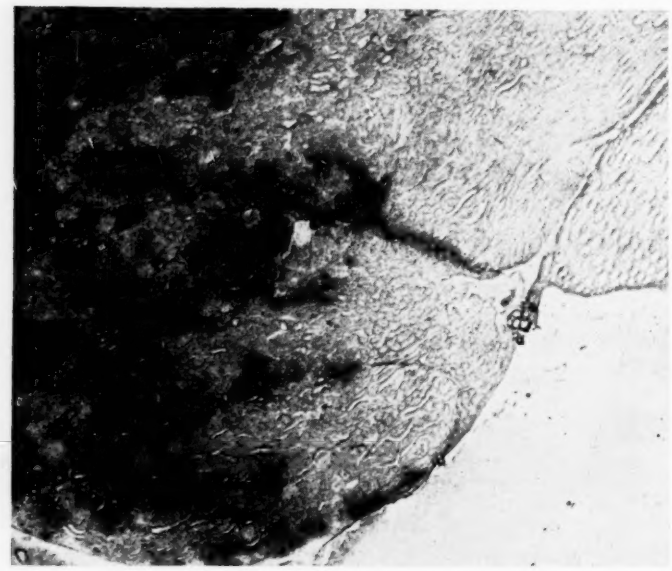

FIG. 5. RADIOAUTOGRAPH OF RAT KIDNEY AFTER I.V. INSULIN-I^{131}
Note the concentration of radioactivity in the proximal convoluted tubules.

the feces. The total radioactivity recovered in the urine and feces in 48 hours approximated 70 per cent of the administered dose.

Renal radioactivity

The high renal concentration of radioactivity with insulin-I^{131} was investigated further. Radioautography (Figure 5) demonstrated that relatively little of the radioactivity was in the medulla and glomeruli, but that it was highly concentrated in the convoluted tubules, particularly the proximal tubules.

Perfusion of the kidney failed to remove appreciable quantities of radioactivity. In this experiment, six rats averaging 255 Gm. in weight were used. In three, the upper abdominal aorta and the right renal artery were tied off 15 minutes after the insulin-I^{131} was given, and the left kidney, with its vein cut, was perfused with 20 ml. of normal saline, through the distal aorta. In the other three animals, the usual procedure was followed, no perfusion was done, but right and left kidneys were counted separately as a control for the previous experiment.

The data in Table I show that practically none (5.1 per cent) of the insulin * was removed from the kidney by perfusion. However, 38 per cent of the non-precipitable material was removed. The over-all reduction of radioactivity, then, was of the order of 13 per cent.

Comparison of distribution of insulin-I^{131} with I^{131}-labeled human serum albumin and NaI^{131}

The possibilities that the distribution might have been related to (a) the I^{131} label, or to (b) the fact that the insulin-I^{131} was a foreign protein, were investigated by studying the distribution of (a) NaI^{131} alone, and (b) I^{131}-labeled human serum albumin (HSA-I^{131}). Eleven rats, averaging 300 Gm. in weight, were treated in the same manner as that used to inject insulin-I^{131}. Four were given NaI^{131} and seven received HSA-I^{131}, the latter in doses of 40 μg. The former were fasted, the latter not.

Rats were sacrificed at 5 and 15 minutes. In Figure 6, the data are plotted along with that of insulin-I^{131}. The renal concentration with insulin-I^{131} was many fold that with either comparison substance. Hepatic concentration was also higher, although not as striking. Blood levels with NaI^{131} and insulin-I^{131} were comparable, but with HSA-I^{131}, were very much higher.

TABLE I

Renal perfusion study 15 minutes after i.v. insulin-I^{131}

(a) Comparison of radioactivity in right and left kidneys
(Neither kidney was perfused)

Animal No.	TCA precipitate			TCA supernatant			Total radioactivity		
	CPM* right kidney	CPM left kidney	Per cent difference	CPM right kidney	CPM left kidney	Per cent difference	CPM right kidney	CPM left kidney	Per cent difference
1	18,610	19,502	4.7	4,451	4,451	0	23,061	23,953	3.7
2	17,088	16,732	2.1	3,959	3,942	0.5	21,047	20,674	1.8
3	17,002	16,200	4.7	5,192	5,192	0	22,194	21,392	3.6

(b) Left kidney perfused; right control

Animal No.	TCA precipitate				TCA supernatant				Total radioactivity			
	CPM right kidney	CPM left kidney	per cent difference	Average per cent diff.	CPM right kidney	CPM left kidney	per cent difference	Average per cent diff.	CPM right kidney	CPM left kidney	per cent difference	Average per cent diff.
4	20,088	18,350	8.7		5,829	3,311	43		25,917	21,661	16.4	
5	13,062	12,440	4.8	5.1	4,119	2,532	39	38.0	17,181	14,972	12.8	13.3
6	12,326	12,108	1.8		4,836	3,231	33		17,162	15,339	10.6	

* CPM = Counts per minute.

Distribution in humans

The data are plotted in Figure 7. In four instances the patients were terminal and the interval from insulin-I^{131} injection to death is recorded, although the tissues were not actually obtained until autopsy some hours later. Tissues were obtained at operation in one case: insulin-I^{131} was given intravenously during a gastrectomy and biopsy specimens for radioactivity were taken 15 minutes later. The distribution pattern in the human showed many resemblances to that in the rat, and data from the rat are included for com-

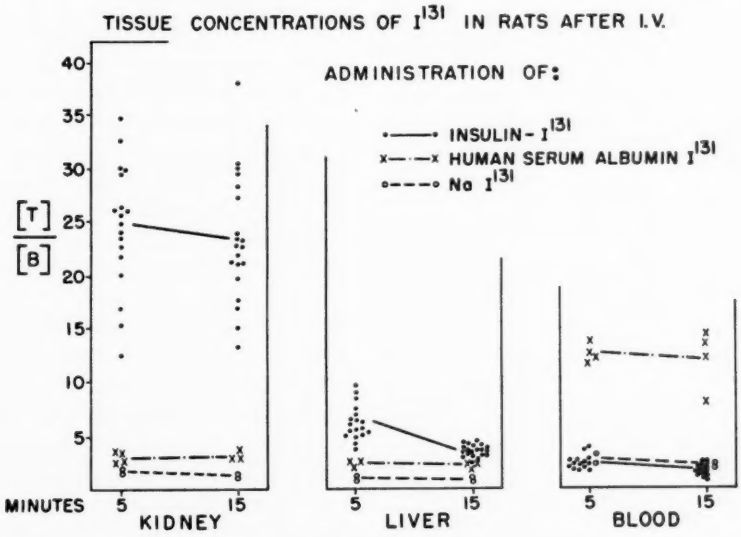

FIGURE 6

Each point represents the total radioactivity in the tissue of one rat. Note that insulin-I^{131} is concentrated markedly in the kidney and to some extent in the liver.

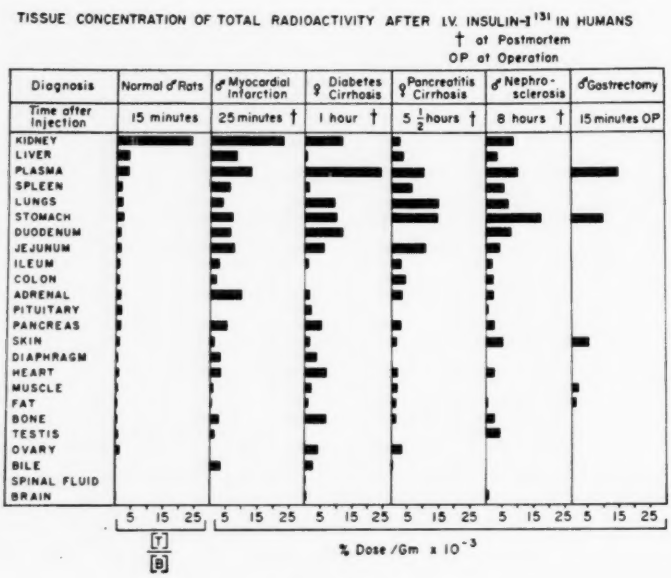

FIGURE 7

Data on rats are included for comparison.

parison. The resemblance was most apparent in the tissues of a man whose demise of a myocardial infarction occurred 25 minutes after the insulin-I^{131} was given. The high concentration in kidney was striking. The gastro-intestinal tract in all instances seemed to have a higher proportion than seen in the rat, but of course, the interval permitted for distribution and breakdown was longer. Muscle, in general, had a low concentration. The liver contained considerable activity except in the patients with cirrhosis, in one of whom it was conspicuously low. Of special note was the low concentration in brain, cerebrospinal fluid, and bile.

DISCUSSION

Iodine is not a normal constituent of insulin. In being labeled, the insulin binds iodide (one atom in 500 of which is radioactive) presumably on the tyrosine and/or histidine groups. Strictly, then, insulin-I^{131} is an insulin derivative, and the data must be treated with this reservation in mind. However, such would be the case with any radioactive preparation since, of course, insulin normally does not contain radioactivity. Although heavy iodination has been demonstrated to inactivate insulin (5), in our experiments there was at the most one iodine atom for each insulin molecule.[8] Bioassay has not shown that this relatively light iodination effected any impairment of physiological activity.

The I^{131} stayed with the protein fraction during chromatographic, electrophoretic, and TCA precipitation experiments, so that in certain physical, as well as physiological properties, insulin-I^{131} behaved as insulin. In tissue, however, there was a rapid increase in the amount of radioactivity in the non-TCA-precipitable fraction. Presumably, as the insulin-I^{131} molecule was broken down, some of the I^{131} was associated with peptide fragments, or amino acids, or was liberated as iodide, and thereby was no longer precipitated by TCA. Accordingly, the relatively short time interval of 15 minutes was used in many of the distribution studies in an effort to allow time for adequate mixing

[8] This figure represents an approximation derived from the data of Ferrebee, Johnson, Mithoefer and Gardella (1). They calculated "two iodine molecules per insulin molecule" (molecular weight 40,000). Actual iodine assays of three of our Abbott preparations have shown one iodine atom per 20 to 40 insulin molecules (molecular weight 20,000).

and yet minimize the overlay of redistribution of labeled breakdown products.

Despite the above evidence that insulin-I^{131} behaved as natural insulin, the premise that the distribution of radioactivity represented non-radioactive insulin or its breakdown products, can only be inferred, pending further biological and biochemical investigation. Present evidence supports this premise however: (a) The distribution of radioactivity was quite different from that following the injection of the label, NaI^{131}, alone. (b) The distribution was unlike that of I^{131}-labeled human serum albumin. This argues against speculation that the distribution represented simply that of any iodinated foreign protein. (c) It is pertinent to compare our observations of the distribution of I^{131}-labeled insulin with similar studies of another low molecular weight protein hormone, ACTH, labeled with I^{131}. With at least three such preparations, Sonenberg (6) has shown notably high concentrations of radioactivity in rat kidney. The physiological counterpart of this is striking in the work of Richards and Sayers (7), in which, giving rat ACTH to rats, and measuring the adrenal ascorbic acid depleting factor extractable from tissues, they found that the kidney had the highest concentration (15 per cent in 15 minutes) of any tissue. This shows a similarity of label and bioassay data in the case of ACTH. As regards bioassay data for insulin, there have been many reports of the extraction of insulin activity from organs other than the pancreas. Best, Jephcott, and Scott (8), however, were unable to duplicate the results and the search has apparently not been pursued. There is, then, no report of a physiological counterpart to the present study, and its need becomes apparent. The ACTH data, nevertheless, tend to support the concept that the label on these hormones does not affect their specificity. It is of interest that radioautographs of renal ACTH-I^{131} (9) closely resemble those of insulin-I^{131}.

There are a number of similarities of tissue distribution between that of insulin-I^{131} and that of other protein hormones. This is striking in the case of I^{131} labeled prolactin as reported by Sonenberg, Money, Keston, Fitzgerald, and Godwin (10), in which the concentrations at 15 minutes in the rat kidney, liver, muscle, plasma and other tissues are almost identical to the findings with insulin-I^{131}. This suggests the possibility of common metabolic pathways apart from the individually specific pathways in target gland sites.

The rapid loss of insulin * from the blood and from all tissues, with the appearance of non-TCA-precipitable I^{131} in rapidly increasing amounts, undoubtedly represent degradation of the insulin-I^{131} molecule. It may be that the rapid breakdown of insulin-I^{131} reflects the transient biological activity of insulin seen clinically. This breakdown may also explain the lack of insulin activity found in the bioassay of extrapancreatic tissues, as noted above. Measurement of the rate of breakdown of insulin-I^{131} offers a valuable tool in the study of insulin metabolism; identification of the breakdown products may enable the degradation and metabolism to be further characterized.

The principal sites of action of insulin have classically included muscle and liver (11). Stadie, Haugaard, and Vaughan have shown insulin "binding" by these tissues, measuring, in the case of muscle, both physiological activity and uptake of labeled insulin (12). This is in accord with the demonstration of our studies that large amounts of injected insulin * go to these tissues (Figure 2). The lack of concentration in red blood cells is in harmony with the lack of effect of insulin on the glycolytic system of blood (13). The absence of appreciable insulin * from the brain is compatible with present evidence that the brain does not require insulin (14). A very small fraction, 1.7 per cent, of the insulin * injected, appeared in the urine in 24 hours. Similarly, bioassay for injected insulin has shown that only about 0.1 per cent of a dose came out in the urine in 24 hours, in man (15).

A ready correlation of the high concentration of renal tubular insulin * to renal physiology is not apparent. An insulinase system in liver, muscle, and kidney has been described by Mirsky and Broh-Kahn (16). The analogy of the renal insulin-I^{131} to renal ACTH-I^{131} has been noted above. It may be pertinent that ACTH is inactivated by kidney slices and homogenates (17).

The data on distribution in humans were obtained in terminal states, and were, in most instances, obtained hours after the insulin-I^{131} was given. Accordingly, there was an overlay of re-

distribution of breakdown products. Nevertheless, the general pattern seems to resemble that in the rat.

SUMMARY

1. Studies using I^{131}-labeled insulin are reported and discussed.

2. Distribution studies in rats showed concentrations of radioactivity to be high in kidney and liver, low in red blood cells and brain, and intermediate in other tissues, including muscle. The distribution pattern was different from that of NaI^{131} and I^{131}-labeled human serum albumin.

3. Perfusion and radioautographic studies showed that the renal radioactivity was bound in the convoluted tubules.

4. The concentrations of total and protein precipitable radioactivity in rat blood and tissues were maximal within 15 minutes after intravenous insulin-I^{131} injection, and thereafter fell, in general at rapid rates. Non-protein-precipitable radioactivity, initially negligible, rapidly increased, as the insulin-I^{131} was broken down. Fifty per cent of the I^{131} was in the urine in 24 hours, but only 1.7 per cent was protein precipitable.

5. Distribution studies in the human showed many similarities to those in the rat.

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THE MECHANISM OF THE OSMOTIC ADJUSTMENT OF BODY CELLS AS DETERMINED *IN VIVO* BY THE VOLUME OF DISTRIBUTION OF A LARGE WATER LOAD[1]

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Since permeability of cell membranes to water has been clearly demonstrated, there are several theoretically possible responses of cells to dilution of the extracellular fluid. Osmotic equilibrium might result from net movement of water into cells in response to extracellular dilution. On the other hand, net movement of water into cells might be averted by: 1) active transport of water out of cells preventing a change in intracellular osmotic activity; 2) reduction of intracellular osmotic activity by means of either osmotic inactivation of intracellular solutes or their extrusion from the cells.

These possibilities were tested by determining the volume of distribution of a large water load which depends on movement of water through the body. The data are compared with the total body water content measured by the dilution of a tracer amount of deuterium oxide which is independent of net movement of water. In the dog the volume of distribution of a large water load was found to approximate closely the total body water content.

METHODS AND PROCEDURE

A. Protocol

Fifteen initial experiments were performed in nine unanesthetized dogs. Two and one-half to five units of Pitressin Tannate in Oil® [4] were given two to sixteen hours before the experiment to reduce the urinary loss of water. After the bladder was emptied by catheterization, the animal was weighed and a control blood sample obtained. A water load of 100 ml. per kilogram of body weight was then administered intravenously at a rate of approximately 20 ml. per minute. The water load was given as a 2½ per cent solution of dextrose, or dextrose and fructose. Blood samples were obtained following the infusion at intervals of three to sixty minutes for five or six hours in three experiments. In the other experiments blood samples were taken 4 to 6 hours after the infusion was completed. Some of the dogs received 0.3 mgm. of atropine and 50 mgm. of Dramamine® to prevent vomiting and salivation.

A second series of ten experiments was performed on dogs either nephrectomized or with ureters ligated. The operation was done under local procaine infiltration (10 to 20 ml. of 1 per cent solution) in two animals and in the remainder under general anesthesia induced and maintained throughout the experiment by the intravenous administration of 25 to 30 mgm. of sodium pentobarbital per kilogram of body weight. Immediately following operation a blood sample for the natural abundance of deuterium was drawn, and 1 to 2 grams of deuterium oxide per kilogram of body weight were given intravenously. Blood was drawn one and one-half and two hours later for determination of deuterium and as control samples before dilution. Dramamine® and atropine were administered as above. One hundred and thirty-two ml. per kilogram of body weight of a 2.5 per cent solution of fructose and/or glucose were administered intravenously at a rate of 30 ml. per minute. This quantity of water was chosen to increase the degree of dilution and thus to minimize the effects of the errors of measurement on the calculated results. The two final blood samples were obtained between 2½ and 3½ hours after the infusion was completed. The ureteral ligatures were removed from four dogs, all of which recovered uneventfully. In one of these the experiment was later repeated.

B. Analytical methods

Plasma sodium and potassium concentrations were measured by flame photometry (1). Total solute concentration of plasma was determined by the freezing point depression measured with a thermistor.[5] Deuterium concentrations were measured by mass spectrometry (2).

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[4] Kindly provided by Dr. A. C. Bratton, Jr., Parke, Davis and Company, Detroit, Michigan.

[5] The apparatus was developed and constructed for us by Fiske Associates, Inc., 44 Bromfield Street, Boston, Mass.

Urea was determined by the microdiffusion technique of Conway (3); glucose by the method of Nelson modified by Somogyi (4); fructose by the method of Rolf, Surtshin, and White (5); chloride by the method of Wilson and Ball (6). Plasma water was measured gravimetrically after drying at 95° to 105° C. to constant weight. Data were analyzed by conventional statistical methods (7).

C. Calculations and analytical error

In the calculations of the last ten experiments the values for sodium, total solutes and deuterium are the averages of repeated determinations made on two separate plasma samples obtained at short intervals, as described above. The errors of the methods were estimated by the analysis of variance of these repeated determinations. The coefficient of variation for a single determination of plasma sodium concentration was 0.95 per cent; of total solute concentration, 0.75 per cent; and of deuterium, 1.55 per cent. As all analyses were done at least in quadruplicate the estimate of the analytical error for the averages used in the calculations was reduced to one-half or less of the above figures for single determinations.

The volume of distribution of the water load was calculated by the following general equation:

$$(1) \qquad V = \frac{C_2 W}{C_1 - C_2}$$

V = volume[6] of distribution of the water load.
C_1 and C_2 = initial and final plasma concentrations of sodium or total solutes.
W = volume of water administered.

Total body water content was calculated as follows:

$$(2) \qquad V_{D_2O} = \frac{18}{20} W_{D_2O} \left(\frac{100 - F}{F} \right)$$

V_{D_2O} = deuterium space (total body water content).
W_{D_2O} = weight of deuterium oxide administered.
F = atoms per cent excess of deuterium in plasma at equilibrium.

The volumes, as calculated, had a minimum standard deviation attributable to propagation of the errors of measurements alone (8) estimated to be: 1.4 to 2.4 per cent of body weight for the sodium calculations, 1.6 to 3.0 per cent of body weight for total solute calculations, and approximately 0.5 per cent of body weight for the deuterium space.

RESULTS

The results obtained in the initial fifteen experiments performed in nine dogs are briefly summarized in Table I. These results were calculated according to Equation 1 with no attempt to correct the data for the small renal losses of water and solute that occurred during the experiments.

[6] No distinction has been made between liters and kilograms of water.

TABLE I

The mean volume of distribution of an intravenous water load in 15 experiments

Mean V_{Na}	= 65.6% of Body Weight
Mean V_{Sol}	= 63.6% of Body Weight
Mean Difference	= 1.9% of Body Weight
Standard Deviation (of Mean Difference)	= 2.3% of Body Weight
t_{14}	= 0.86
p	= 0.4

Analysis of variance reveals no significant difference between the mean volumes of distribution of the water load calculated from changes in concentration of plasma sodium (V_{Na}) and total solute (V_{Sol}), but the standard deviation for the differences between V_{Na} and V_{Sol} in individual experiments was large (8.9 per cent body weight). The results approximate the expected value for total body water in the dog.

Because of the considerable discrepancy between V_{Na} and V_{Sol} in individual experiments, ten further studies were done. To reduce errors resulting from renal losses of water and solutes, either the dogs were nephrectomized or their ureters were ligated immediately prior to the experiment.

Figure 1 shows the effects of a water load on the total solute, sodium, glucose and urea concentrations of plasma in a nephrectomized dog. Equilibrium of distribution of the water load occurred within two hours. The plasma urea concentration slowly increased during the study as might be expected in a nephrectomized animal. The plasma glucose concentration was still elevated above the control levels at the end of the experiment.

The data for the last ten experiments are presented in Table II. The quantity of water administered was chosen to cause a large dilution of the body fluids with minimal toxic effects. The serum sodium concentrations after the water load are in a range not uncommonly seen clinically. In eight experiments there occurred a slight rise in plasma urea concentration in spite of the dilution. This accumulation of urea is attributable to the suppression of renal function. In all experiments the blood glucose level was still elevated at the time the last blood samples were drawn. This

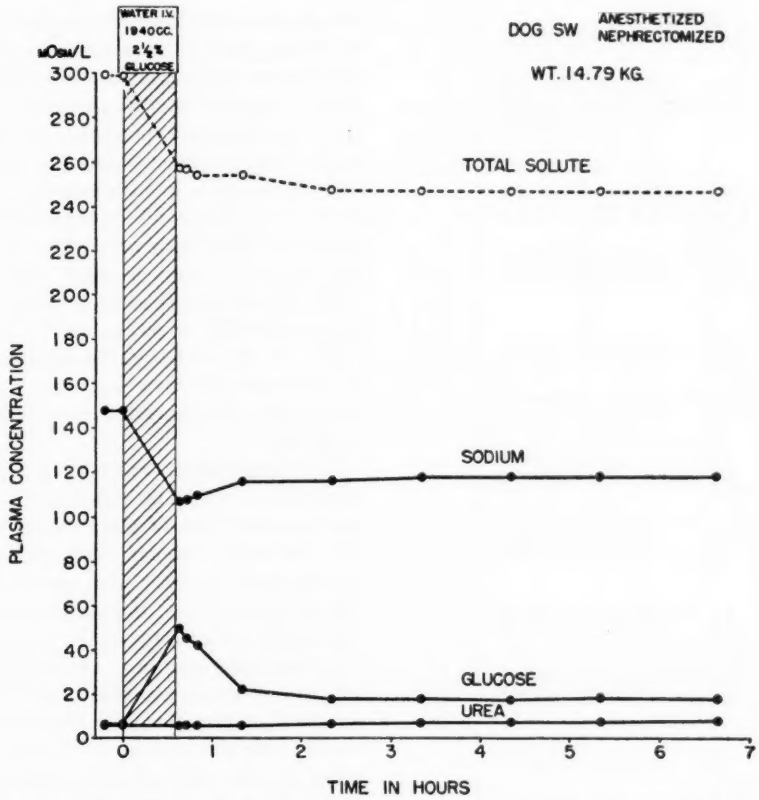

FIG. 1. CHANGES IN TOTAL SOLUTE, SODIUM, GLUCOSE AND UREA CONCENTRATIONS IN PLASMA FOLLOWING THE INTRAVENOUS ADMINISTRATION OF A LARGE WATER LOAD TO A NEPHRECTOMIZED DOG

It is evident that the plasma concentrations of sodium and total solutes had achieved constant values by 2½ hours following termination of the infusion. In this experiment the plasma glucose remained elevated after the infusion (see text).

was true even when fructose alone was used in the infusing solution. The concentrations of fructose in the plasma, however, never rose more than 1.3 mM per L. The persistent hyperglycemia probably reflects reduced glucose tolerance resulting from the meat diet used prior to the experiment.[7] The serum potassium concentrations decreased slightly in all but two experiments. In the two experiments in which serum potassium concentration rose the changes were too small to reflect significant extrusion of intracellular potassium in response to extracellular dilution.

[7] Three days of glucose administration prior to the experiment in subsequent studies abolished the prolonged hyperglycemia noted in the present results.

Table III shows the volume of distribution of the large water load calculated from changes in plasma sodium (V_{Na}) and total solute (V_{Sol}) concentrations, respectively. The total body water content before dilution, determined as the volume of distribution of deuterium oxide (V_{D_2O}), is also shown. To facilitate comparison, all results are expressed as per cent of body weight. The plasma urea concentrations have been subtracted from the total solute concentrations used to calculate V_{Sol} in Tables III and IV to correct for the accumulation of urea during the experiments. This correction is justified because urea is known to be freely distributed over total body water.

The results in Table III are subdivided into

TABLE II

Data for the second series of experiments

Exp.	Wgt.	Water load	Plasma concentrations before and after dilution (mM/L.)										Plasma water %		Deuterium oxide	
			Sodium		Total solutes		Urea		Glucose		Fructose		Potassium			
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Given	Atoms
	L.				*(mOsm/L.)*										*(gm./ml.)*		*gm.*	*% excess*
1	10.22	1.37	151.2	125.9	305.3	258.0	12.0	13.3	4.2	5.3			4.5	4.1	.9263	.9393	18.228	.244
2	16.75	2.20	147.7	121.5	311.1	261.3	17.7	17.4	6.8	10.9	.11	.17	4.3	3.4	.9404	.9467	17.278	.153
3	14.99	1.98	148.1	121.0	299.6	252.0	13.7	13.5	6.9	7.6	.11	1.39	4.4	4.8	.9436	.9516	16.646	.153
4	11.53	1.50	146.9	122.3	289.4	247.5	5.6	7.8	5.6	7.7	.12	.54	4.0	3.2	.9357	.9467	16.617	.201
5*	11.00	1.45	150.3	123.3	303.8	254.4	7.9	9.5	9.2	11.2	.19	.47	3.1	3.0	.9371	.9444	17.806	.222
6	11.83	1.58	149.2	119.1	293.8	242.8	8.0	8.5	5.7	11.7	.17	.88	4.2	4.7	.9380	.9479	17.915	.239
7	15.75	2.08	146.4	116.7	288.5	242.4	4.6	5.4	6.1	14.2	.12	1.40	4.0	3.2	.9283	.9420	16.512	.166
8*,†	11.55	1.50	150.2	121.5	298.0	257.3	7.1	8.5	8.1	16.9	.14	.93	3.9	3.2	.9328	.9453	17.711	.221
9†	14.63	1.94	144.8	119.2	288.1	249.7	7.3	9.0	6.1	13.3	.09	.71	4.5	4.0	.9379	.9434	17.233	.160
10	9.2	1.31	148.0	120.2	290.3	249.7	7.1	8.7	6.4	15.5	.16	.41	3.3	3.0	.9263	.9443	20.314	.302

* Repeated experiments on the same animal.
† Unanesthetized.

two groups according to the magnitude of the rise in plasma glucose concentration (ΔG) that occurred between the initial and the final blood specimens. Although in all experiments, V_{Sol} is larger than V_{Na}, the five experiments in Group I show an excellent agreement among V_{Na}, V_{Sol}, and V_{D_2O}. The discrepancies in each experiment within this group are of the magnitude expected from the analytical and sampling errors. The value of ΔG for this group of results is small. In Group II of Table III, the discrepancies between V_{Na} and V_{Sol} are considerably larger, as are the values of ΔG. Figure 2A shows the relationship between ΔG and the difference between V_{Sol} and V_{Na} for all experiments. The correlation coefficient, 0.88, is significant.

The accumulation of glucose or any other solute during the course of an experiment will result in a final concentration of total solutes which is too high and will minimize the expected dilution of plasma total solutes. This would cause falsely high values for V_{Sol}. If glucose accumulates in an osmotically active form in the extracellular fluid, it not only will result in an over-estimation of V_{Sol}, but also would be expected to reduce V_{Na}. The osmotically active glucose in the extracellular fluid would cause the movement of some intracellular water to the extracellular compartment. This would dilute the plasma sodium in excess of the dilution produced by the water load and V_{Na} would be underestimated. An attempt has, therefore, been made to correct the results of Table III for the measured changes in plasma glucose.

In Table IV, V_{Na} and V_{Sol} have been corrected on the assumption that the rise in glucose concentration occurred over a volume of 30 per cent of body weight. This volume of distribution of diffusible glucose in the dog has recently been reported by Searle, Strisower, and Chaikoff (9). Details of this calculation are presented in the Appendix; it should be noted that the volume of distribution assumed for glucose does not enter into the correction applied to the calculation of V_{Na}. The values of V_{D_2O} are again presented to facilitate comparison. The correction for plasma water content and the Donnan effect, though insignificant, was also applied to V_{Na} (see Appendix).

The correction for glucose abolishes the significant correlation of $V_{Sol} - V_{Na}$ and ΔG (Figure

TABLE III

Uncorrected volume of distribution of administered water

(Expressed as per cent of body weight)

Experiment	ΔG†	V_{Na}	V_{Sol}	V_{D_2O}
Group I				
1	1.09	66.7	67.5	65.6
2	4.09	60.8	64.6	60.6
3	0.75	58.9	66.4	65.2
4	2.13	64.6	70.8	64.5
5*	1.95	60.3	63.3	65.5
Group II				
6	6.04	52.8	60.8	57.0
7	8.14	52.0	66.7	56.8
8*	8.78	54.8	76.8	62.4
9	7.17	61.5	79.7	66.2
10	9.05	61.7	81.5	65.7

* Repeated observations on the same animal.

† ΔG is the difference in concentration of plasma glucose before and after dilution.

V_{Na} and V_{Sol} are the volumes of distribution of the water load calculated from changes in plasma sodium and total solute concentrations, respectively.

V_{D_2O} is the total body water content determined from dilution of deuterium oxide.

TABLE IV

Volume of distribution of administered water corrected for glucose, Donnan effect, and plasma water content

(Expressed as per cent of body weight)

Experiment	V_{Na}	V_{Sol}	V_{D_2O}
1	66.2	66.0	65.6
2	64.1	60.5	60.6
3	59.6	64.8	65.2
4	61.9	67.9	64.5
5	67.0	60.6	65.5
6	57.4	55.9	57.0
7	54.0	59.4	56.8
8	56.5	67.0	62.4
9	63.1	71.0	66.2
10	62.6	71.1	65.7
Mean	61.2	64.4	63.0

Standard deviation of differences between means

$V_{Sol} - V_{Na} = 1.80$; $t = 1.76$; $p > .05$

$V_{D_2O} - V_{Na} = 0.96$; $t = 1.76$; $p > .05$

$V_{Sol} - V_{D_2O} = 1.03$; $t = 1.43$; $p > .05$

2B). It is apparent from Table IV that the calculation of the volume of distribution of a large water load is essentially the same whether estimated from the dilution of serum sodium or total solutes. Furthermore these values closely approximate the total body water content (V_{D_2O}).

DISCUSSION

Studies with deuterium oxide have clearly demonstrated that cell membranes are permeable to

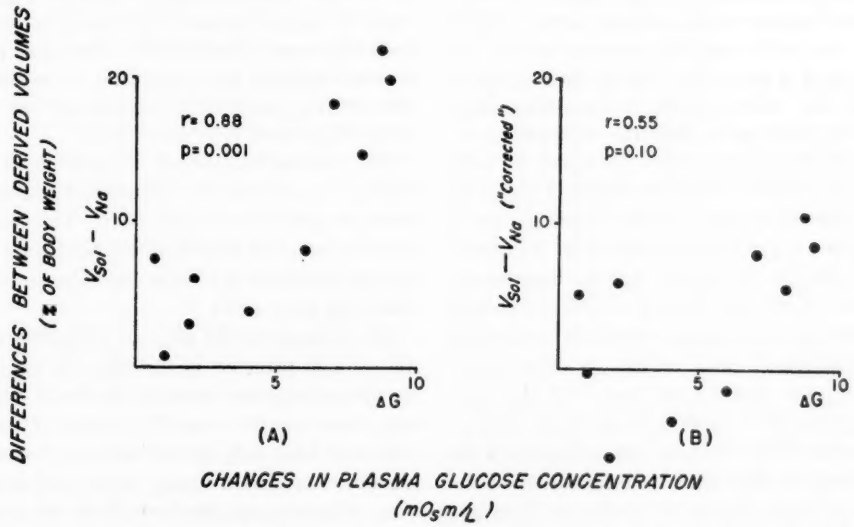

FIG. 2. THE CORRELATION BETWEEN CHANGES IN PLASMA GLUCOSE CONCENTRATIONS, ΔG, AND THE DIFFERENCES BETWEEN V_{Sol} MINUS V_{Na} (A), AND THE CORRECTED VALUES OF V_{Sol} MINUS V_{Na} (B)

The correlation is significant in A, but not significant in B.

water. The distribution of a small quantity of deuterium oxide throughout total body water is dependent upon the movement of individual molecules and does not require net passage of water across cell membranes. Hence, the knowledge that deuterium oxide equilibrates with total body water yields no information about the osmotic adjustments of the cells when a large quantity of water is introduced into the body.

The results of the present study strongly suggest that the volume of distribution of a large water load is equal to the total body water content. Thus *in vivo* the osmotic adjustment of the great majority of body cells involves net movement of water into cells so that the water load appears to be evenly distributed throughout total body water.

This interpretation of the present findings seems contradictory to the conclusions of Robinson and McCance (10, 11), who observed that respiring rat kidney slices maintained essentially constant weight in media of varying concentrations, whereas slices whose respiration had been inhibited swelled in these media. They concluded that the cells are normally hypertonic to their environment and that inward diffusion of water is counteracted by an equal and active transport of water out of the cells. Such a mechanism would prevent net movement of water into cells and the apparent volume of distribution of a water load should then approximate only the volume of the extracellular fluid. The present findings exclude the widespread occurrence of such active water transport by cells though it is possible within the errors of measurement of this study that a single tissue or organ might behave in the manner claimed by Robinson. However, Mudge has shown (12) in experiments with kidney slices that shifts of electrolytes as well as of water may occur across renal cell membranes making Robinson's data subject to other interpretations. The findings of Opie (13) that several tissues swell *in vitro* in media hypertonic to normal extracellular fluid are not pertinent to the present study as they give no information of the response of these tissues to dilution of their environment *in vivo*. Furthermore this worker fails to explain why such swelling of tissues does not occur *in vivo*.

The present study yields results representing the average response of the body cells to dilution. It is, therefore, not possible to deny that some cells take up more than their quota of the water load whereas other cells take up less. It seems unlikely, however, that certain cells are diluted more than the average in an amount just sufficient to yield a volume of distribution for various water loads apparently equal to total body water.

If enough intracellular solute were extruded from the cells, V_{Sol} might equal total body water. V_{Na}, however, would be significantly lower unless the extruded solute were sodium. An attempt was made to determine whether sodium was extruded from bones or cells. The ratio of sodium to chloride in bone and most cells is much higher than in the extracellular fluid. If sodium were extruded into the extracellular fluid one might expect a greater dilution of chloride than of sodium. In two instances the dilution of sodium and of chloride were identical. In the other experiments the chloride dilution was highly variable and the discrepancies between sodium and chloride dilution correlated closely with the volume of gastric secretions which accumulated during the experiments. It seems improbable that sodium would move out of bone and cells in all experiments in an amount just sufficient to increase V_{Na} to the value of V_{D_2O} in spite of differences in the water loads that were administered. For these reasons, it seems unlikely that extrusion of sodium from cells or bone contributes significantly to the osmotic adjustment to a water load.

The osmotic inactivation of intracellular solutes might be an alternative adjustment requiring no net movement of water into cells. This possibility must be excluded since it would result in a volume of distribution of the water load significantly less than total body water.

The present results give no indication whether the osmotic pressure is the same or different in the intracellular and extracellular fluids. In either case, however, the even distribution of the water load over total body water indicates that the concentration ratio is the same before and after dilution. Osmotic equality throughout the total body water would be the simplest explanation consistent with the present results. This view has been accepted and clearly stated by Peters (14) and is in agreement with recent cryoscopic measurements

of various tissues by Conway and McCormack (15).

This study is complementary to earlier work of Hetherington (16) and Eggleton (17) and recent studies of Wolf and McDowell (18). These workers added hypertonic saline to the extracellular fluid and measured the apparent volume of body water that diluted the administered salt. The average results of Hetherington, Wolf, and McDowell and those of Eggleton, as recalculated by Conway and McCormack (15) yielded fair approximations of total body water content.

SUMMARY AND CONCLUSIONS

In twenty-five experiments, eighteen dogs were given large water loads under conditions which minimized or eliminated renal losses. The volume of distribution of the water load was calculated from the changes in plasma concentrations of sodium and total solutes. In ten experiments total body water content was simultaneously determined by the dilution of deuterium oxide.

The volume of distribution of the water load as determined from the dilution of plasma solute (64.4 per cent of body weight) and sodium (61.2 per cent) concentrations was equal to total body water content (63.0 per cent) within the limits of experimental error. The findings indicate that *in vivo* the great majority of the body cells adjust to acute dilution of the extracellular fluid by a net movement of water into the cells so that the administered water load is distributed evenly over total body water content.

ACKNOWLEDGMENTS

The authors wish to thank Miss Denise Girod and Miss Mineko Sasahara for their assistance in this study. A Supply Grant from the National Institutes of Health in support of a Public Health Service Postdoctorate Research Fellowship to one of us (E. P. T.) was used in this study.

APPENDIX

W = Water load in liters.

V_1 = Volume of distribution of the water load.

$V_2 = V_1 + W$.

α = Coefficient of dilution, equals $\frac{V_1}{V_1 + W}$, after equilibrium.

m_1, m_2 = Fraction of V_1 and V_2, respectively, containing osmotically active glucose.

n_1, n_2 = Fraction of V_1 and V_2, respectively, containing sodium at its concentration Na_1 and Na_2.

Na_1, Na_2; G_1, G_2; S_1, S_2 = Concentrations in plasma of sodium, glucose and total-solutes-minus-urea before and after dilution. Na and G concentrations are expressed as millimoles per liter; S, in milliosmoles per liter.

K = The factor by which Na_2 is corrected for overdilution as a result of accumulation of glucose.

f = The factor by which plasma sodium concentration is corrected for plasma water content and the Donnan effect.

I. *Correction of V_{Sol} for change in glucose*

The total amount of solutes in m_2V_2 is the same as the amount in m_1V_1 plus the added glucose which is not metabolized:

$$m_2V_2S_2 = m_1V_1S_1 + (m_2V_2G_2 - m_1V_1G_1) \tag{3}$$

It is assumed that the glucose concentrations, G_1 and G_2, are uniform throughout their respective volumes of distribution, m_1 and m_2.

The increase in concentration, ΔS, of total solutes resulting from this unmetabolized glucose is:

$$\Delta S = \frac{m_2V_2G_2 - m_1V_1G_1}{V_2} \tag{4}$$

Replacing m_2 by its value obtained from (*3*):

$$\Delta S = m_1\alpha\left[\frac{S_1 - G_1}{S_2 - G_2}G_2 - G_1\right] \tag{5}$$

Thus the general equation (*1*) permits calculation of V_{Sol} corrected for change in glucose:

$$V_1 = \frac{W}{\frac{S_1}{S_2 - \Delta S} - 1} \tag{6}$$

II. *Correction of V_{Na} for change in glucose and for plasma water content and the Donnan factor*

The increase in osmotically active glucose in the extracellular fluid may be expected to draw water from cells. This will result in overdilution of the plasma sodium which may be corrected by a factor:

$$K = \frac{n_2V_2}{n_1V_1} \tag{7}$$

As $\frac{m_2}{m_1} = \frac{n_2}{n_1}$, equations (*3*) and (*7*) may be combined to yield:

$$K = \alpha\frac{S_1 - G_1}{S_2 - G_2} \tag{8}$$

Plasma sodium concentration was corrected for plasma water content and for the Donnan effect assuming one-fourth of the extracellular fluid to be intravascular. The Donnan factor, d, of 0.96 was accepted for a plasma protein concentration of 6 per cent and $(1 - d)$ was considered to vary directly with plasma protein concentration. Plasma protein concentration was calculated from plasma water content (gm. per ml.) (19):

$$f = \frac{0.334}{\text{Plasma Water}} + 0.671 \tag{9}$$

Thus the general equation (*1*) permits calculation of

V_{Na} corrected for change in glucose and for plasma water content and the Donnan effect:

$$(10) \qquad V_{Na} = \frac{W}{\frac{f_1 Na_1}{K f_2 Na_2} - 1}$$

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THE EFFECTS OF ALKALOSIS UPON KETONE BODY PRODUCTION AND CARBOHYDRATE METABOLISM IN MAN

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Available evidence from animal investigation suggests that changes in pH can alter carbohydrate and ketone metabolism. Guest and Rawson (1) observed a distinct inverse relationship between blood sugar content and pH in rats. In the fasting animal, alkalosis was found to enhance ketosis and decrease liver glycogen (2). In human studies, Beumer and Soecknick (3) and Porges and Lipschütz (4) noted the early appearance of acetonuria in patients on subcaloric diets treated with sodium bicarbonate.

The present study was undertaken to define some of the metabolic changes attendant upon the acute administration of large amounts of alkali to normal and diabetic individuals in the postabsorptive state. Because of the known ketogenic activity (5) of acetic acid and the key position occupied by the 2-carbon fragment "acetate" in the intermediary metabolism of various foodstuffs (6), it was decided to compare the effect of alkalosis induced by sodium acetate to that of sodium bicarbonate.

EXPERIMENTAL PROCEDURES AND METHODS

An infusion of an isotonic solution consisting of 20.5 gm. of sodium acetate dissolved in 1600 cc. of distilled water was administered intravenously at a constant rate over a two hour period in all experiments. Eleven of the subjects, four medical students and seven patients hospitalized for minor ailments, were young adult males without evidence of disturbances of hepatic function or carbohydrate metabolism. Of the nine male diabetic subjects selected with ages ranging from 25 to 55 years, two patients, J. Z. and A. C., required no insulin, being controlled by diet alone. Both had undergone leg amputations for peripheral vascular disease six months prior to study. The diagnosis of diabetes mellitus was well substantiated before operation by the usual clinical and laboratory criteria. At the time of investigation both were found to be normoglycemic. The remaining seven subjects received no insulin for 2 to 5 days prior to observation. Several developed various degrees of diabetic ketosis during this period. One had mild associated acidosis. All subjects were maintained on diets containing adequate calories, protein, fat, and 250 grams of carbohydrate for at least one week prior to the study. Tests were routinely performed at 8 A.M. with the subjects in the postabsorptive state.

Analyses were performed before and at 120, 180, 240, and, in some instances, 280, 320, and 360 minutes after the onset of the infusion. Serum electrolytes were measured by methods previously described in publications from this laboratory (7). Citric acid in the serum was determined by the method of Natelson, Pincus, and Lugovoy (8). Blood was analyzed for total ketones, *i.e.*, the sum of acetone, acetoacetic acid, and β-hydroxybutyric acid by the method of Michaels, Margen, Liebert, and Kinsell (9), glucose by the iodometric titration method of Somogyi (10), and pyruvic acid by the technique of Friedemann and Haugen (11). When significant degrees of ketonemia were present, pyruvic acid filtrates were treated in a manner described by Lardy (12). Recoveries for glucose, ketones, citric and pyruvic acids ranged from 95 to 99 per cent in all instances.

Observations were made in two diabetic subjects, utilizing the hepatic vein catheterization technique of Bradley, Ingelfinger, Bradley, and Curry (13). Hepatic blood flow was determined by the bromsulphalein method as outlined by these authors.

Simultaneous samples of peripheral venous and arterial blood were obtained through indwelling Cournand needles placed in the left antecubital vein and brachial artery. Analyses of blood for glucose and ketones were performed in triplicate. The standard deviation of replicate samples for glucose at the 100 mgm. per cent level was $\pm$ 1 mgm.; for ketones at 1–2 mgm. per cent, $\pm$ 0.064; and at 20 mgm. per cent, $\pm$ 0.305 mgm. per cent.

Finally, eight paired control studies were performed in two consecutive days on four normal and four diabetic subjects. In one, sodium acetate, and in the other an equimolar solution of sodium bicarbonate was used.

RESULTS

Changes in acid-base balance

The administration of either sodium acetate or sodium bicarbonate solutions to normal and dia-

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TABLE I

Serum CO_2 and chloride response to the intravenous administration of equimolar quantities of Na acetate and $NaHCO_3$ in normal and diabetic subjects *

	Na Acetate						$NaHCO_3$					
	Normals			Diabetics			Normals			Diabetics		
Time	No.	CO_2	Cl	No.	CO_2	Cl	No.	CO_2	Cl	No.	CO_2	Cl
min.		*mEq./L.*			*mEq./L.*			*mEq./L.*			*mEq./L.*	
0	11	25.2 ±0.177	95.1 ±0.812	8	24.7 ±0.453	94.2 ±0.988	6	25.6 ±0.486	95.3 ±1.36	6	24.2 ±0.583	91.4 ±1.88
120		30.5 ±0.291	88.9 ±0.945		29.5 ±0.517	88.7 ±0.877		31.3 ±0.482	89.3 ±1.53		29.1 ±0.538	87.1 ±1.63
240		28.8 ±0.389	90.5 ±1.90		27.7 ±0.692	90.3 ±1.06		29.8 ±0.408	90.5 ±1.65		28.3 ±0.772	89.3 ±0.808

* The mean and standard error.

betic subjects produced comparable degrees of metabolic alkalosis. Table I summarizes the changes observed in all studies including the paired experiments. Alkalosis was maximal at the termination of the infusion (120 minutes) and still persisted at 240 minutes, but to a lesser extent. Simultaneously, the chloride concentration of the serum decreased to a roughly equivalent degree in all cases.

Ketones

Two diabetic patients, L. R. and D. B., were subjected to hepatic vein catheterization prior to the administration of sodium acetate (Figures 1, 2). The initial ketone concentration in the blood of L. R. was slightly elevated. D. B. was in moderate diabetic ketosis at the onset of the procedure. The splanchnic ketone output which was determined by multiplying the hepatic vein-arterial

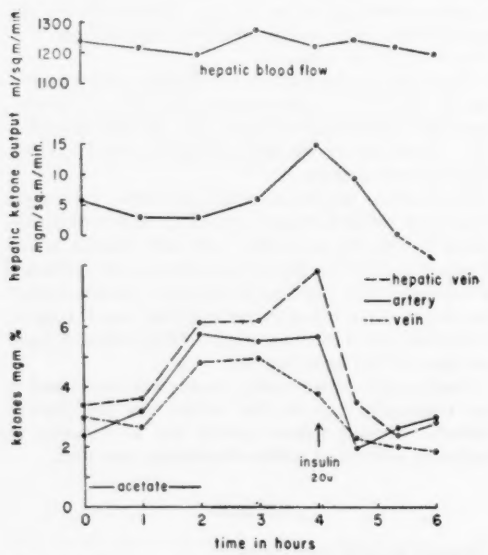

FIG. 1. PATIENT L. R. WITH MILD DIABETES. THE EFFECT OF ACETATE AND INSULIN ON HEPATIC BLOOD FLOW AND HEPATIC KETONE PRODUCTION

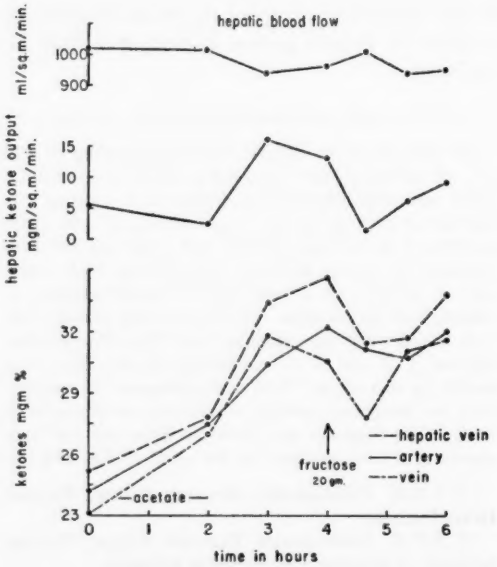

FIG. 2. PATIENT D. B. WITH SEVERE DIABETES. THE EFFECT OF ACETATE AND FRUCTOSE ON HEPATIC BLOOD FLOW AND HEPATIC KETONE PRODUCTION

(HV-A) ketone difference by the estimated hepatic blood flow was quantitatively similar in both instances. Ketone production rose from 5 mgm. per M^2 per minute to 15 mgm. per M^2 per minute at 180 (D. B.) and 240 minutes (L. R.) after the onset of the infusion. The greatest HV-A differences were apparent at these points. The largest peripheral arterial-venous (A-V) differences were noted at 240 minutes. The hepatic blood flow remained relatively constant throughout.

Eighty minutes after the intravenous administration of 20 units of regular insulin to subject L. R., splanchnic ketone production as evidenced by HV-A differences fell, approaching zero. The concentration of ketones in the hepatic vein, peripheral artery, and vein fell to initial levels. These effects persisted until the termination of the experiment.

Likewise, 40 minutes after the rapid intravenous injection of 20 grams of fructose to patient D. B., a sharp depression in splanchnic ketone output was noted. The peripheral A-V difference increased significantly at this point, indicating increased peripheral ketone utilization. However, these effects were only fleeting, for 80 and 120 minutes later ketone production was again approaching precarbohydrate injection levels. Similar changes are reflected in the ketones of the hepatic vein, peripheral artery, and vein.

The intravenous infusion of 100 grams of fructose over a 60 minute period to a patient in diabetic ketosis, not treated with acetate, resulted in a simliar decline followed by a secondary rise above preinjection ketone concentrations (14).

The response of blood ketones to intravenously administered sodium acetate and sodium bicar-

TABLE II

The blood ketone response to intravenous administration of sodium acetate and sodium bicarbonate in normal and diabetic subjects

Normals	Time in minutes 0	120	180	240
	mg. %			
Na Acetate				
F. G.	1.6	1.8	1.6	2.9
S. D.	2.0	3.0	4.7	4.3
G. M.	2.1	2.7	3.3	4.0
H. D.	2.0	2.0	2.9	3.6
T. H.*	2.0	2.0	1.6	2.9
E. F.*	1.5	2.0	1.9	2.8
F. J.*	1.5	1.6	2.7	2.7
K. H.*	1.4	1.2	1.4	2.5
Mean	1.8	2.0	2.5	3.2
Standard Error	±0.102	±0.58	±1.13	±0.235
$NaHCO_3$				
R. S.	1.6	1.6	1.7	2.1
L. L.	1.7	2.4	1.9	2.0
T. H.*	1.8	1.7	1.7	1.4
E. F.*	2.4	1.4	1.2	0.9
F. J.*	1.4	1.1	0.9	0.8
K. H.*	1.6	1.5	1.4	1.1
Mean	1.8	1.6	1.6	1.4
Standard Error	±0.143	±0.179	±0.163	±0.229

Diabetics	Time in minutes 0	120	180	240
	mg. %			
Na Acetate Mild Diabetics				
L. R.	3.0	4.8	5.0	3.8
A. C.	1.3	1.7	2.8	2.9
F. H.*	2.0	2.8	3.8	3.7
L. D.*	3.2	2.8	3.4	4.4
J. S.*	0.7	1.7	2.5	1.9
Mean	2.0	2.8	3.5	3.5
Standard Error	±0.480	±0.565	±0.04	±0.43
Na Acetate Severe Diabetics				
H. B.	16.0	25.3	26.6	29.2
D. B.*	23.0	26.9	31.8	30.6
D. B.	10.7	14.1	17.2	16.4
L. B.*	27.4	38.8	40.5	46.7
Mean	19.3	26.2	29.0	30.7
Standard Error	±3.7	±5.0	±5.0	±6.2
$NaHCO_3$ Mild Diabetics				
F. H.*	4.1	3.5	5.5	3.1
L. D.*	3.2	2.3	3.4	2.7
J. S.	1.9	2.3	2.4	2.5
H. B.	3.2	2.3	6.1	7.0
Mean	3.1	2.6	4.3	3.8
Standard Error	±0.45	±0.30	±0.72	±1.07
$NaHCO_3$ Severe Diabetics				
D. B.*	35.3	39.3	42.3	42.2
L. B.*	23.2	26.7	29.4	33.0

* Paired studies.

bonate in normal and diabetic subjects are presented in Table II. In both single and paired observations in the normal group, following acetate infusions, blood ketones increased slightly, but significantly ($p < 0.01$) and consistently. In the control series receiving bicarbonate there were no similar changes. Indeed, in the majority of subjects, ketone concentrations diminished slightly and progressively. A comparison of the mild diabetics at 0 and 240 minutes shows a significant increase in ketones following acetate infusions, but a borderline or insignificant increase following bicarbonate. In severe diabetics, ketones increased appreciably following infusions of either acetate or bicarbonate. The absolute rise was greatest in those individuals who were initially in diabetic ketosis.

Glucose

The effects of acetate on the splanchnic glucose metabolism of the two diabetic subjects, L. R. and D. B., are shown in Figures 3 and 4. In both individuals, splanchnic glucose output decreased strikingly. In patient L. R. this was most marked at 120 and 240 minutes. Two hours after the intravenous administration of 20 units of insulin, the blood sugar of this patient had fallen approximately 40 mgm. per cent with an associated increase in splanchnic glucose production. The action of insulin on peripheral blood sugar concentrations and splanchnic glucose output was similar to that observed by Bearn, Billing, and Sherlock (15). In subject D. B. glucose production fell precipitously. The splanchnic bed began to remove glucose from the circulation, producing negative values for HV-A. This was most pronounced at the completion of the acetate infusion and persisted to a lesser degree for an additional 120 minutes. However, with the passage of time, there was a tendency for the glucose concentration in the hepatic vein to rise again above that in the artery. At 240 minutes the rapid intravenous injection of 20 grams of fructose induced an outpouring of hexose into the blood stream by the liver. This phenomenon was dissipated within a 40 minute period, and only after peripheral concentrations began to descend again was an upward trend in output seen.

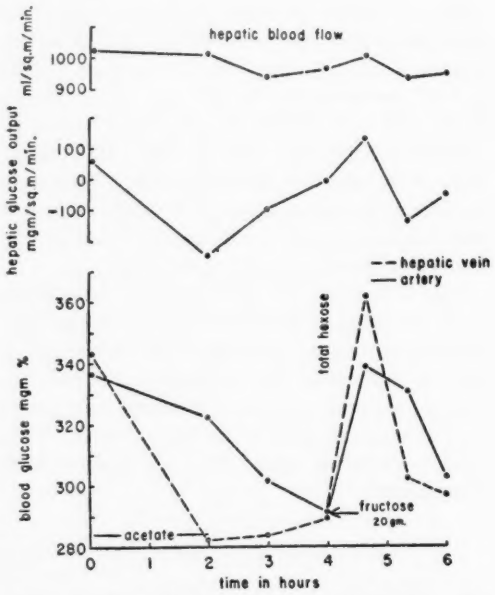

FIG. 4. PATIENT D. B. WITH SEVERE DIABETES. THE EFFECT OF ACETATE AND FRUCTOSE ON HEPATIC BLOOD FLOW AND HEPATIC GLUCOSE PRODUCTION

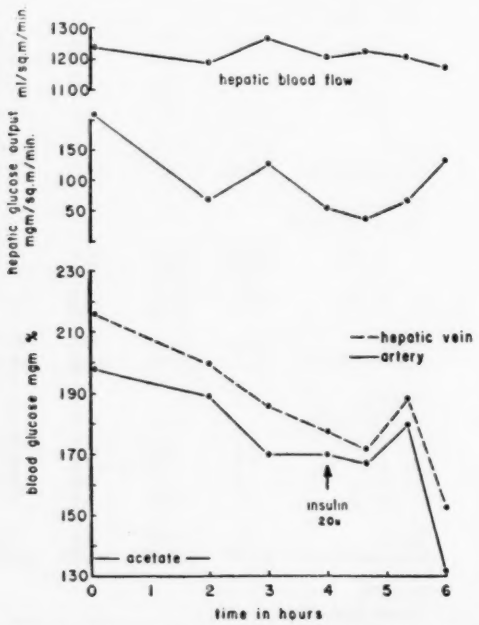

FIG. 3. PATIENT L. R. WITH MILD DIABETES. THE EFFECT OF ACETATE AND INSULIN ON HEPATIC BLOOD FLOW AND HEPATIC GLUCOSE PRODUCTION

TABLE III

The response of blood glucose to intravenous administration of sodium acetate and sodium bicarbonate in normal and diabetic subjects

Normals	Time in minutes 0	120	180	240	Diabetics	Time in minutes 0	120	180	240
	mg. %					*mg. %*			
Na Acetate					Na Acetate				
W. J.	84	82	84	82	A. P.	201	186	184	174
F. G.	80	82	83	77	L. R.	215	193	178	168
S. D.	67	67	72	70	E. B.	184	164	163	167
G. M.	63	69	73	74	H. B.*	237	203	200	203
H. D.	63	62	71	77	D. B.*	437	382	393	389
T. H.*	74	73	76	82	D. B.	339	323	297	292
E. F.*	78	76	81	81	F. H.*	212	190	176	162
K. H.*	75	70	78	81	L. D.	172	149	143	141
F. J.*	76	73	80	73	J. Z.†	86	87	88	86
					A. C.†	92	88	91	94
Mean	73	73	77	78	Mean	217	196	191	187
Standard Error	±2.17	±2.54	±1.60	±1.47	Standard Error	±33.3	±29.4	±28.9	±28.7
$NaHCO_3$					$NaHCO_3$				
R. S.	84	86	87	84	H. B.*	241	177	199	186
L. L.	102	106	110	110	D. B.*	436	415	379	367
T. H.*	75	74	71	70	F. H.*	207	157	162	164
E. F.*	82	81	80	84	L. D.*	218	182	180	175
K. H.*	79	77	80	72	J. Z.†	104	101	107	100
F. J.*	72	78	71	69	A. C.†	83	77	79	92
Mean	82	84	83	82	Mean	215	185	183	181
Standard Error	±4.32	±4.73	±5.69	±6.33	Standard Error	±51.7	±49.2	±43.0	±40.5

* Paired studies.

† Normoglycemic at time of study, had substantiated diagnosis of diabetes, and required insulin prior to leg amputations for peripheral vascular disease.

Table III shows the changes observed in the glucose of the blood following the infusion of acetate and bicarbonate to normal and diabetic individuals. The blood sugar of the normal subjects did not change appreciably in single and paired studies after the administration of either solution. In the diabetic group, however, at the termination of the experiment, mean decreases of 30 and 34 mgm. per cent below initial concentrations were noticed after the injection of acetate and bicarbonate, respectively. There was no distinct correlation between the height of the initial blood sugar and the degree of depression at the termination of the experiment.

Citric acid

Citric acid concentrations in the blood increased significantly in both normal ($p < 0.02$) and diabetic subjects ($p < 0.02$) at 240 minutes following the infusion of acetate (Table IV). Small increments noted after the bicarbonate solution were not statistically significant. In this group, at 180 minutes, two subjects showed peak increases of similar magnitude to those seen in the acetate treated series. However, these increments were followed by slight declines at the 240 minute pe-

TABLE IV

Blood citric acid response to the intravenous administration of Na acetate and $NaHCO_3$ in normal and diabetic subjects *

Time	Na Acetate				$NaHCO_3$			
	Normals		Diabetics		Normals		Diabetics	
	No.	Citric acid	No.	Citric acid	No.	Citric acid	No.	Citric acid
0	5	2.30 ±0.157	7	2.28 ±0.098	6	2.09 ±0.204	6	2.33 ±0.094
120		2.42 ±0.295		2.12 ±0.105		1.91 ±0.234		2.09 ±0.229
180		2.68 ±0.241		2.59 ±0.375		2.25 ±0.273		2.39 ±0.114
240		2.80 ±0.085		2.78 ±0.141		2.39 ±0.249		2.58 ±0.081

* Mean and standard error of mean.

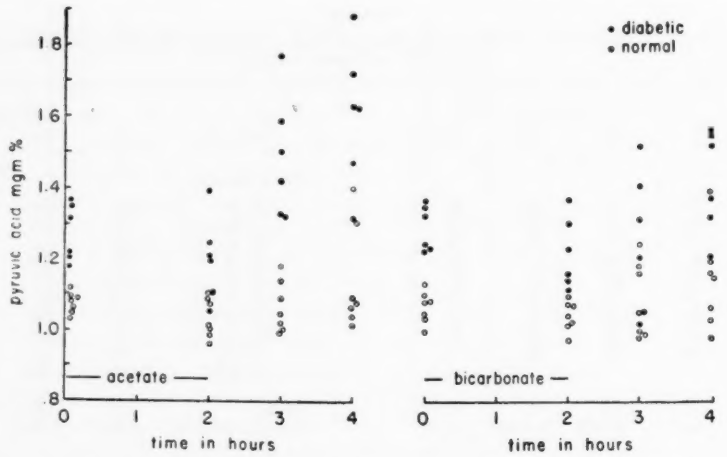

FIG. 5. THE EFFECTS OF ACETATE AND BICARBONATE ADMINISTRATION ON BLOOD PYRUVIC ACID CONCENTRATION IN NORMAL AND DIABETIC SUBJECTS

riod. The initial values of both groups fell within the normal range for blood citric acid concentration.

Pyruvic acid

The administration of acetate or bicarbonate to normal subjects (Figure 5) usually resulted in a slight decline of blood pyruvate at the termination of the experiment. In two instances, using either solution, pyruvate concentrations rose at this point. A more definitive pattern was noted in the diabetic group of patients (Figure 5). Generally, slight depressions in pyruvic acid at the end of the infusion period were followed by subsequent increases above initial concentrations. In the acetate treated group this elevation (12 to 38 per cent) appeared earlier, was higher and more consistent than that noted in subjects receiving bicarbonate.

The patients with diabetes mellitus exhibited slightly higher pre-injection concentrations than the normal group.

DISCUSSION

Acetate as such is not found in the blood stream under normal conditions. The rapidity with which this compound is metabolized in the mammalian body has been demonstrated by Harper, Neal, and Hlavacek (16). Following the rapid intravenous injection of C^{14} labeled acetate (4.8 mM per Kilo, in 1 to 2 mins.) to dogs, these investigators noted the quick appearance and maximal concentration of C^{14} in the CO_2 of the expired air within 30 minutes after administration. During the same period, the formation of new $C^{14}O_2$ ceased as the tagged acetate rapidly disappeared from the blood. This was taken as presumptive evidence for utilization of this compound. If the data obtained from these experiments are applied to our studies, it can be assumed that at the rate of administration of 15 grams of exogenous acetic acid (20.5 grams CH_3COONa or 3.5 mM per Kilo) over 120 mins., the metabolism of 2C fragments was almost instantaneous. However, the amount of acetate that could be given was limited by the associated sodium load. Under these circumstances the alkalosis that was produced was comparable to that induced by equimolar amounts of $NaHCO_3$. With the rapid utilization of the acetate radical, the associated sodium ions became available for combination with CO_2 and H_2O. The effect in each instance, therefore, was an increase in the concentration of sodium bicarbonate in the serum. Since it is virtually impossible to differentiate the metabolic effects of acetate "loading" from those of concomitant alkalosis, it seems reasonable to conclude that the results obtained in this study probably were related to alterations in the pH of body fluids *per se*.

Alkalinization ($NaHCO_3$ or Na acetate) in the diabetic subject was associated with a fall in blood sugar and an accentuation of pre-existing ketosis (Tables II, III). In the two diabetic patients (Figures 3–4) subjected to hepatic vein catheterizations, splanchnic glucose production decreased and hepatic ketone output increased following the administration of sodium acetate. From the results of previous animal investigations on the effects of alkalosis on carbohydrate, protein, and fat metabolism (2), it appears that these changes are, for the most part, directly related to a decrease in the transformation of protein to glucose. Since ketosis varies inversely with the availability of carbohydrate, the amount of fat converted to ketone bodies in the diabetic becomes largely dependent upon the extent and rate at which these catabolic processes take place. In the acetate experiments, it is conceivable that the addition of 2C fragments could alter total body metabolism. Fifteen grams of acetate given over two hours would represent an appreciable fraction of the caloric requirement during this period of time. For example, if this rate of administration were continued over 24 hours, 180 grams of acetate could provide over one-half the total fat and carbohydrate intake per day in equivalent weight of carbon chains. Two carbon fragments are rapidly converted into ketone bodies in the uncontrolled diabetic. Although ketone bodies can readily be utilized to meet, in part, the energy requirements of the body, it is improbable that this effect alone could account for the diminution in the production of glucose from various sources, since similar results were noted following bicarbonate administration.

The lack of appreciable changes in glucose concentration noted during alkalosis in the normal subjects, and in the two diabetic patients, J. Z. and H. C., who became normoglycemic following leg amputations for obliterative peripheral vascular disease, remains unexplained. It may be hypothesized that the observed fall occurs only in the presence of either overproduction of glucose from noncarbohydrate sources or inadequate stores of liver glycogen. However, the latter seems unlikely, for in a normal subject fasted for 48 hours and whose liver glycogen was presumably depleted, the blood sugar remained relatively unchanged while ketosis was enhanced with alkalosis (14).

In eight paired studies ketone concentrations in the blood of normal subjects failed to increase following the administration of $NaHCO_3$ (Table II). The individuals in these experiments were in the postabsorptive state. No prior stimulus for ketosis existed. However, it has been noted that in the presence of depleted carbohydrate stores, alkalosis induced by either $NaHCO_3$ (3, 4) or hyperventilation (3, 17) will accentuate ketosis. In the latter situation, respiratory alkalosis was associated with an actual decrease in serum bicarbonate. In the acetate treated group of normals the slight but significant increase in ketone bodies probably represents the result of condensation of small excesses of preformed acetate.

It has long been known that shifts in pH toward alkalinity are associated with substantial increments of citric acid in blood and urine (18). The efficiency of the reaction acetoacetate + oxaloacetate → citric acid may therefore be considerably increased during alkalosis. Thus in the subjects receiving acetate, the mechanisms for the increased production of citric acid may occur singly or in combination. First, it may be an effect of alkalosis alone; second, it may be due to the availability of excesses of acetate or acetoacetate in the presence of intracellular oxaloacetate. The slight fall in citric acid noted in several studies coincident with maximal alkalosis at the termination of the infusion at 120 minutes probably represents a distribution phenomenon.

Very little is known concerning the pathways of pyruvate metabolism in man. The somewhat variable changes that were noted in blood pyruvate can not be explained at this time in the light of present knowledge.

SUMMARY

1. The administration of large amounts of acetate as the sodium salt to normal and diabetic subjects results in the production of an alkalosis comparable to that induced by equimolar solutions of $NaHCO_3$.

2. In normal subjects there were no changes in blood glucose concentrations following the infusion of acetate or bicarbonate.

3. The administration of sodium acetate to diabetic subjects caused depressions in splanchnic glucose output and peripheral glucose concentrations. These effects were associated with simul-

taneous increases in splanchnic ketone production and peripheral blood ketones. Diabetics treated with bicarbonate usually exhibited similar changes in peripheral glucose and ketone levels.

4. The effect of alkalosis *per se* on carbohydrate metabolism is discussed.

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AN OPEN-CIRCUIT HELIUM METHOD FOR MEASURING FUNCTIONAL RESIDUAL CAPACITY AND DEFECTIVE INTRAPULMONARY GAS MIXING[1]

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In pulmonary disease the inspired air is often distributed unequally throughout the lung. The volume of the poorly aerated regions of the lung and their rate of ventilation are of importance to the clinician but quantitation of these values has been difficult.

The situation was much clarified in 1950 by Robertson, Siri, and Jones (1) who demonstrated that an unequally ventilated lung behaves as though it consisted of a number of subdivisions of smaller size, each of which is homogeneously ventilated at its own particular rate. Preliminary work which led to the same concept was also reported at this time by Fowler, Cornish, and Kety (2). By continuously following the course of nitrogen elimination from the lungs of a subject breathing oxygen, it was possible to characterize these lung subdivisions in terms of size and ventilation rate. This information describes an unequally ventilated lung in a way which permits useful, quantitative predictions of its behavior. However, these studies required complex instrumentation.

It is the purpose of the present report to describe a simple method of quantitating unequal pulmonary gas mixing along these lines, and of measuring the functional residual capacity and correcting it for errors resulting from unequal mixing. The method employs an open-circuit arrangement, using helium as the test gas. By this means large, slowly ventilated lung subdivisions have been found in persons with emphysema; in severe cases the mixing inequality seems great enough to interfere seriously with conventional open-circuit residual air measurements.

METHODS

The subject first breathes a 50 per cent helium, 50 per cent oxygen mixture for 15 minutes, as shown in Figure 1A. This is usually long enough to achieve a steady state with respect to helium concentration throughout the lung. The subject is then switched at the end of a normal expiration to tank oxygen (Figure 1B), and the expired gas is thereafter diverted into a collecting system which has been prewashed with oxygen. For at least the first 7 minutes all the expired gas is collected in a 100 liter Douglas bag for later measurement of volume and helium concentration. The expired gas stream is continuously analyzed for helium by a katharometer, as shown in Figure 1. Approximately 250 ml. per minute is passed through a drier and carbon dioxide absorber (Ascarite), through the katharometer, through a gas-tight Thiberg pump, and then is returned to the system down-stream from the sampling point.

The katharometer system consists of a Gow-Mac[3] RCT thermal conductivity cell with a sealed reference cell, and an RCT control unit. The output of this system is measured by a Leeds and Northrup potentiometer having a range of 0 to 64 millivolts. This arrangement allows analysis of helium-oxygen mixtures up to about 3 per cent helium. Calibration of the equipment with known mixtures of tank helium and tank oxygen within this range yielded a linear relationship between helium concentration and the electrical potential developed by the katharometer, with a standard deviation from regression of 0.04 per cent helium. After an adequate warm-up period the katharometer is very satisfactorily stable. At the pump rate of 250 ml. per minute and with the Ascarite train, the assembly gives a 95 per cent response to change in helium concentration within 20 seconds. In addition, a few seconds delay is introduced by the volume of gas in the corrugated tubing between mouthpiece and sampling point. This volume amounts to about 1 liter and serves the purpose of mixing expired gas sufficiently to allow a smooth wash-out curve. Because of this slight delay and its sensitive response to helium, the katharometer is suited for measuring relatively slow phases of helium excretion from the lung which may occur after the initial, rapid wash-out. For clinical purposes this terminal, slow phase of helium excretion is of primary importance.

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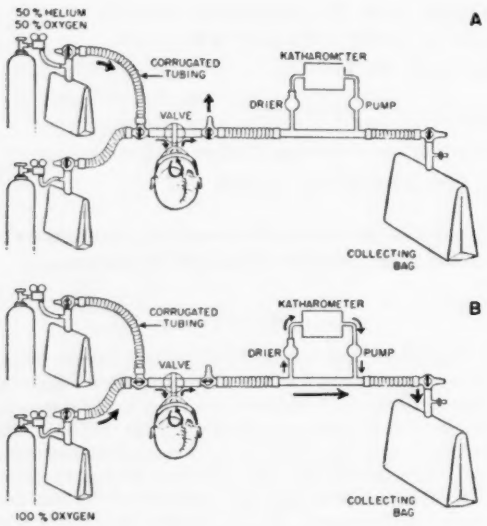

FIG. 1. DIAGRAM OF THE APPARATUS

Section A shows the direction of gas flow during the initial equilibration period. Section B shows the flow during the wash-out period.

CALCULATIONS

Calculation of functional residual capacity from volume and helium concentration of gas in collecting bag

Except for subjects with severe inequalities in intrapulmonary mixing, the functional residual capacity (FRC), which is the lung volume at the end of a quiet expiration, can be calculated from the volume and concentration of helium collected in the Douglas bag during the first 7 minutes. As in other open-circuit methods, the basic procedure consists of determining the total amount of test gas which has been washed out of the lung into the bag, and dividing this volume by the concentration of the test gas in the lung before the wash-out. This quotient, with appropriate corrections for temperature and water vapor, is the functional residual air. In these calculations the following symbols are used in accordance with standard terminology (3):

F_{He} = true fractional concentration of helium in the collecting bag.

F'He = fractional concentration of helium in the collecting bag as indicated by the katharometer after absorption of CO_2.

F_ECO_2 = fractional concentration of CO_2 in expired air. This is assumed to be .025. The error which this assumption may introduce in the measurement of the FRC will be discussed below.

V(ATPS) = Volume, in ml., of gas in the collecting bag at ambient temperature and pressure, saturated.

V(BTPS) = Volume of gas in collecting bag at body temperature and pressure, saturated.

AT = Ambient temperature, Centigrade.

BP = Barometric pressure, mm. Hg.

P_{H_2O} = Water vapor pressure, mm. Hg, at ambient temperature.

FRC = functional residual capacity at BTPS, in ml.

F_AHe = Fractional concentration of helium in alveolar gas after breathing the helium-oxygen mixture for 15 minutes.

F_AO_2, F_ACO_2, F_AN_2 are the respective fractional alveolar concentrations of O_2, CO_2 and N_2.

F_IO_2, F_IHe are the fractional concentrations of inspired O_2 and He.

R = the respiratory quotient.

The helium concentration in the collecting bag, as determined by the katharometer, is corrected for the error introduced by absorbing CO_2 before analysis:

$$F\,He = F'He(1 - F_ECO_2) = 0.975\,F'He \quad (1)$$

The gas volume in the collecting bag is corrected to BTPS:

$$V(BTPS) = \frac{V(ATPS)(BP - P_{H_2O})}{(BP - 47)} \cdot \frac{(273 + 37)}{(273 + AT)} \quad (2)$$

The expression for the FRC is:

$$FRC = \frac{(FHe)(V(BTPS))}{F_AHe} \quad (3)$$

To obtain the actual value of the FRC, it is necessary to know the value of F_AHe. This can be estimated without serious error for lungs or regions of lungs where gas mixing is good enough to reach virtual equilibrium with the inspired helium mixture by the end of the 15 minute breathing period. Where this is not the case, a correction can be made, as described later. F_AHe is, of course, quite close to F_IHe when mixing is

adequate. The estimation of F_AHe is as follows:

$$F_AHe = 1.00 - F_AO_2 - F_ACO_2 - F_AN_2 \quad (4)$$

From the data of Jones (4) on nitrogen excretion while breathing oxygen, F_AN_2 is estimated to be .005 after 15 minutes of breathing the helium-oxygen mixture. F_ACO_2 is taken to be .060. Allowing a respiratory quotient of 0.80 and expressing this conventionally in terms of inspired and alveolar gas concentrations (3), it is possible to solve for F_AO_2:

$$R = \frac{F_ACO_2(1 - F_IO_2)}{F_IO_2(1 - F_ACO_2) - F_AO_2}$$

Given the specific values of $R = 0.80$, $F_ACO_2 = .060$, and $F_IO_2 = F_IHe = 0.500$, then $F_AO_2 = .432$. Substituting these values in equation (4) yields

$$F_AHe = .503.$$

This value which is very close to F_IHe can be substituted in equation (3) to allow calculation of the FRC.

Several assumptions have been made, but errors in these do not introduce large errors into the determination of the FRC. Specifically, an error of .010 in F_2CO_2 introduces a fractional error of about .01 in the FRC; errors of .010 in F_AN_2, F_ACO_2, and F_IHe (and F_IO_2) will each produce fractional errors of approximately .02 in the FRC; and variation in R between 0.70 and 1.00 will result in a fractional error of no more than .01 in the FRC. F_IO_2 can, of course, be determined by analysis of the commercially supplied mixture. Errors caused by inexactness in the other assumptions, most of which are concerned with the estimate of F_AHe, are not likely to be serious. The helium concentration was measured in 10 "end-expiratory" alveolar samples from eight normal subjects after breathing the same 50 per cent He–50 per cent O_2 mixture for 15 minutes. The mean helium concentration was 50.0 per cent with a standard deviation of 0.5 per cent.

Two corrections must be applied to the estimate of FRC as given in equation (3). First, the volume of the instrumental dead space and the connecting tubing which contributes its helium to the collection, in the present case a total of 180 ml., must be subtracted. In the second place, account must be taken of the error introduced by solution of helium in the body and its subsequent elimination during the wash-out period. Taking into consideration the solubility of helium in body tissues (5, 6), the data of Jones (4) on elimination of inert gases from the body, and the duration of the absorption and excretion periods in the present procedure, it is assumed that about 75 ml. of helium will be excreted from the blood during the wash-out period. This will contribute 150 ml. to the FRC. Accordingly, the quantity of 330 ml. is subtracted from the expression for FRC, and the final expression is:

$$FRC = \frac{(FHe)(V(BTPS))}{0.5} - 330 \quad (5)$$

The reproducibility of the results obtained by the analytical system has been tested by repeated measurements of the volume of a glass bottle which served as a test lung. The bottle was filled with the standard helium-oxygen mixture, and the mixture was then washed into the collecting bag by a stream of oxygen. The volume of the bottle, measured by its water capacity, was 2.20 liters. Eleven successive volume determinations by the helium method yielded a mean of 2.32 liters with a standard deviation of .04 liters. Accordingly, the method gave quite reproducible results, but the mean was slightly above the true value.

Analysis of the terminal wash-out curve

After the first 1½ to 2 minutes of the wash-out period, the helium concentration in the expired air of most subjects will fall to about 3 per cent and thereby come into the analyzing range of the katharometer system. The concentration is then recorded every 15 or 20 seconds until it has fallen to 0.05 per cent, after which the analysis is abandoned. When intrapulmonary gas mixing is grossly uneven, these data can be used for two purposes: for estimating the volume and ventilation rate of the most slowly ventilated lung spaces, and for applying a correction to the FRC calculated from the 7 minute gas collection.

a) Volume and ventilation rate of the "slow component" of the lung volume

As found by Robertson, Siri, and Jones (1) the excretion of an inert gas from lungs with uneven mixing proceeds as though the lung consists of a number of subdivisions each of which is venti-

lated homogeneously at its own rate. The total excretion rate, R_T, at a given time will then be the sum of these individual excretion rates:

$$R_T = R_1 + R_2 + R_3 + \cdots R_s,$$

where R_s is the contribution of the subdivision with the slowest excretion rate.

The general behavior of a system of this kind has been described elsewhere (4), but it is convenient to present here the features pertinent to the present situation, specifically the way in which an inert gas will be washed out of a single homogeneously ventilated subdivision:

Let: $\dot{V}_T$ = the total minute ventilation of the subject, in ml.
V_s = volume of the subdivision in ml. (BTPS)
$\dot{V}_s$ = ventilation rate of the subdivision in ml. per min. (BTPS)
t = time in minutes
Q_s = amount of helium, ml., (BTPS), in V_s at time t. At $t = 0$, $Q_s = Q_{so}$
$k_s = \frac{\dot{V}_s}{V_s}$. This ratio of ventilation to volume is the "turn-over rate"
R_s = excretion rate of helium in ml. (BTPS) per min. from the subdivision at time t
F_EHe_s = the fractional concentration in the total expired air of helium from this particular subdivision, or $\frac{R_s}{\dot{V}_T}$. At $t = 0$, $F_EHe_s = F_EHe_{so}$

For present purposes $\dot{V}_s$ can be considered to proceed as a continuous, rather than intermittent flow. Accordingly,

$$\frac{dQ_s}{dt} = -\frac{\dot{V}_s Q_s}{V_s} = -k_s Q_s$$

Integration yields:

$$\ln Q_s = -k_s t + \ln Q_{so} \qquad (6)$$

Since $R_s = k_s Q_s$, equation (6) can be expressed as

$$\ln R_s = -k_s t = \ln R_{so},$$

and since $R_s = (F_EHe_s)(\dot{V}_T)$,

$$\ln F_EHe_s = -kt + \ln F_EHe_{so} \qquad (7)$$

Equation (7) describes the contribution made by the subdivision to the helium concentration of the expired gas from the whole lung at any time t after the start of the wash-out. This time-concentration curve will be a straight line on a semi-logarithmic plot. This property permits a simple graphical solution of the equation when a number of experimental points are available. The points are plotted on semi-logarithmic paper with F_EH_s on the ordinate and time on the abscissa, and a straight line is drawn through the points. The intersection of this line with the ordinate yields F_EH_{so}, the concentration of helium at zero time. The value of k is most easily obtained from the "half-time" formula, the half-time ($t\ \frac{1}{2}$) being the time in minutes required for any given value of F_EHe_s to decrease by one-half. This time is determined by inspection of the experimental line. From equation (7), $\ln \frac{F_EHe_s}{F_EHe_{so}} = -k_s t$; therefore at $t\ \frac{1}{2}$, $\ln \frac{1}{2} = -k_s t\ \frac{1}{2}$, or

$$k_s = \frac{.693}{t\ \frac{1}{2}} \qquad (8)$$

If F_EHe_{so} and k_s are determined in this way, and in addition the total minute volume of ventilation, $\dot{V}_T$, is known, then the ventilation rate, $\dot{V}_s$, and volume, V_s, of the subdivision can be determined.

$$R_{so} = (\dot{V}_T)(F_EHe_{so}) = \dot{V}_s \frac{Q_{so}}{V_s}$$

Re-arranging,

$$\dot{V}_s = \frac{(\dot{V}_T)(F_EHe_{so})}{Q_{so}/V_s} \qquad (9)$$

The denominator of this expression is the concentration of helium in the subdivision at the beginning of the wash-out period. In the present procedure this is taken to be 0.50 throughout the lung, as noted above. Knowing $\dot{V}_s$, the value of V_s is determined from the relation $V_s = \frac{\dot{V}_s}{k_s}$.

The use of the present analytical system in measuring ventilation rate and volume of a slow subdivision from the helium wash-out curve was tested on bottles which served as model lungs. The volume and "ventilation rates" of the bottles were known.

Figure 2 shows the wash-out points obtained from two bottles of known volumes and "venti-

lation rates," and Figure 3 shows the result of combining the emergent gas streams and analyzing them together. From the wash-out curve and the total "ventilation rate" a reasonable estimate can be made of the volume and flow rate of the slow bottle.

In the case of an unevenly ventilated lung, the contribution of the most slowly ventilated subdivision is identified as the terminal, straight-line portion of the wash-out curve on semilogarithmic paper. If desired this can be subtracted from the remaining curve and the next slowest subdivision identified.

The question arises whether excretion of helium from the blood significantly affects the terminal portion of the wash-out curve. Since half the normal subjects show no evidence of a slow subdivision, it appears that the present method is not sensitive enough to be much affected by excretion from the blood. In four normal subjects attempts were made to measure helium excretion from the blood after equilibration periods of 15 and 60 minutes followed by 2 minutes of hyperventilation to clear the lungs. After the 15 minute period, the mean excretion rate was 3.3 ml. at 5 minutes and 2.0 ml. at 7 minutes; after the 60 minute period, the rates were about 30 per cent greater. The rates are less than expected from Jones' data (4), but the present method is inexact for these quantities. Such amounts will not significantly affect the present measurement of slow lung spaces.

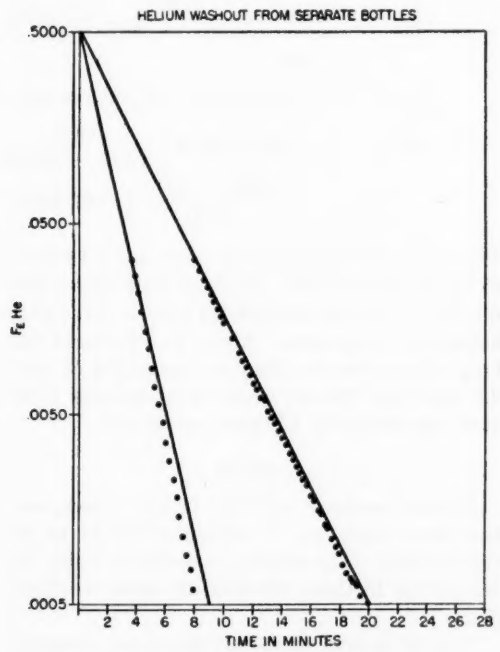

FIG. 2. COMPARISON OF THEORETICAL WITH ACTUAL COURSE OF HELIUM WASH-OUT FROM BOTTLES VENTILATED AT A CONSTANT RATE BY A STREAM OF OXYGEN

The theoretical courses are indicated by the lines and the experimentally found values by the points. F_EHe is the fractional concentration of helium in the wash-out gas. In both cases the bottles contained .50 helium in oxygen at the start. The right-hand line is that of a bottle with a volume of 2300 ml. and a ventilation rate of 800 ml. per min. The left hand line is that of a bottle with a volume of 2100 ml. and a ventilation rate of 1600 ml. per minute.

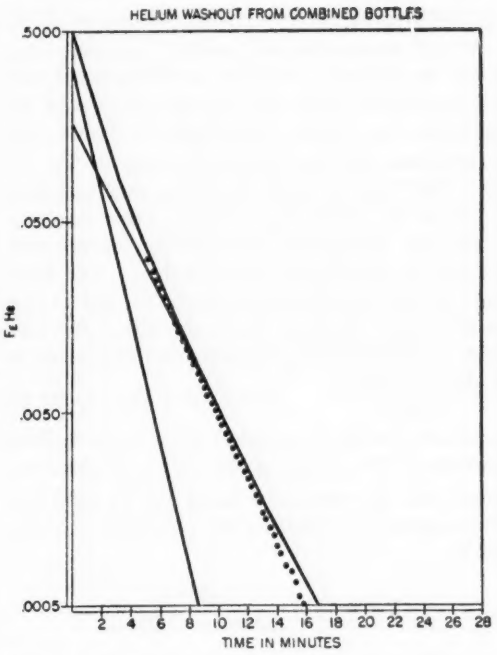

FIG. 3. THE EFFECT OF COMBINING THE EMERGENT STREAMS FROM THE BOTTLES OF FIGURE 2

Volumes and ventilation rates are as before. The straight lines are the calculated contributions of the two bottles to the helium concentration of the combined outflow which is represented by the curved line. The points are the experimental values found for helium concentration in the combined stream. Graphical analysis, as described in the text, predicts for the slow bottle a volume of 2500 ml. and a ventilation of 910 ml. per minute. The actual values are 2300 ml. and 800 ml. per minute.

b) Correction of the slow subdivision and the FRC

Where intrapulmonary mixing is grossly uneven, the slowest ventilated subdivision will not fill completely with the helium mixture in 15 minutes, nor empty completely in 7 minutes. Corrections for the resultant errors in V_s, $\dot{V}_s$ and FRC can be made from the wash-out line of the slow subdivision. These corrections embody the assumption that helium enters and leaves the lungs with equal facility. The fraction of full equilibration which V_s will attain with a new inspired gas composition by any time t after beginning to breathe the mixture is $\frac{F_EHe_{so} - F_EHe_{st}}{F_EHe_{so}}$. Accordingly, the extent to which the subdivision could fill with the helium mixture in 15 minutes is obtained from this fraction, and the correction factor for V_s (and $\dot{V}_s$) is its inverse. Denoting the corrected volumes as V_{sc}:

$$V_{sc} = (V_s)\frac{F_EHe_{so}}{F_EHe_{so} - F_EHe_{s15}}$$

The FRC is corrected by subtracting the fraction of V_s which would have been delivered into the bag in 7 minutes and adding V_{sc}. Denoting the corrected FRC as FRC_c:

$$FRC_c = FRC + V_s\left(\frac{F_EHe_{so}}{F_EHe_{so} - F_EHe_{s15}} - \frac{F_EHe_{so} - F_EHe_{s7}}{F_EHe_{so}}\right)$$

Much the greater part of the error in FRC results from failure to wash well in 7 minutes, rather than from poor filling in 15. The method of correcting the 7 minute FRC has been tested in several subjects with impaired mixing by collecting the expired gas for prolonged periods. The results, presented in Table I, appear to support the procedure.

Sample calculation

The wash-out curve of a 50 year old man with emphysema is presented in Figure 4.

FRC: $V(BTPS) = 92{,}500$ ml.

$FHe = .0312$

$FRC = \frac{(.0312)(92{,}500)}{0.50} - 330 = 5450$ ml.

V_s: $\dot{V}_T = \frac{V(BTPS)}{7} = 13{,}200$ ml.

$t_{\frac{1}{2}} = 3.5$ minutes

$k_s = \frac{.693}{3.5} = 0.20$

$F_EHe_{so} = .0300$

$\dot{V}_s = \frac{(13{,}200)(.0300)}{0.50} = 790$ ml. per min.

$V_s = \frac{790}{0.2} = 3950$ ml.

V_{sc}, FRC_c:

$F_EHe_{s15} = .0016$

$F_EHe_{s7} = .0075$

$V_{sc} = 3950\ \frac{.0300}{.0300 - .0016} = 4170$ ml.

$\dot{V}_{sc} = (0.2)(4170) = 830$ ml. per min.

$FRC_c = 5450 + 3950\left(\frac{.0300}{.0300 - .0016} - \frac{.0300 - .0075}{.0300}\right) = 6630$ ml.

The correction has added approximately 1200 cc. to the 7 minute FRC, of which only about 200 ml. $(V_{sc} - V_s)$ is required for failure to fill with helium in 15 minutes. About two-thirds of the lung volume has an effective ventilation of only 0.8 liters per minute, while the remaining third gets approximately 12 liters per minute.

RESULTS

Measurements of the FRC in the seated position were made on 15 normal males 22 to 28 years of age. The results, presented in Table II, are similar to those obtained by other methods (7).

Typical normal wash-out curves are shown in Figures 5 and 6. Ventilation appears homogeneous in Figure 5, within the limitations of the method, but a small slow space is apparent in Figure 6. The prolonged wash-out curve of a subject with emphysema is presented in Figure 4.

The ventilation pattern may change strikingly in some subjects. This is particularly evident in asthmatic patients following treatment with bronchodilator drugs or on spontaneous improvement.

TABLE I

Effect of correcting FRC in severe emphysema

Subject	Total collecting time (*min.*)	FRC at 7 minutes (*liters*)	FRC, Total time (*liters*)	Corrected FRC (FRC_c) (*liters*)	$V_{ac}-V_a$* (*liters*)	k_a	V_{ac} (*liters*)
C. L.	18	3.64	3.87	3.93	0.00	0.20	1.10
B. S.	15	4.49	5.16	5.48	0.21	0.15	1.92
H. B. (1)	32	3.44	4.07	5.16	0.69	0.09	2.56
H. B. (2)	27	3.65	4.37	5.07	0.44	0.12	2.74
G. H.	20	3.98	5.75	6.43	0.68	0.14	5.30
A. T.	28	4.10	4.90	5.49	0.43	0.13	2.78
W. W.	41	3.77	5.59	6.69	1.10	0.10	4.75

* $V_{ac}-V_a$ is the estimated error resulting from failure of the lung to fill completely with helium in the 15-minute equilibration period. Prolonging the wash-out time cannot correct this error. Subtracting the quantity $V_{ac}-V_a$ from the corrected FRC, which appears in the column immediately preceding, yields the FRC value which should be obtained by a very prolonged wash-out. Inspection of the table indicates that the values actually obtained ("FRC, Total time") approach the predicted value quite closely in most cases.

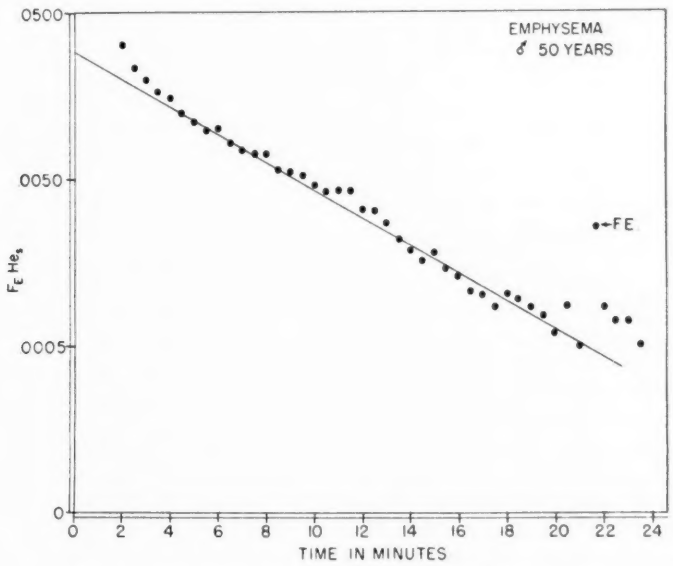

FIG. 4. HELIUM WASH-OUT CURVE OF A 50 YEAR OLD MAN WITH EMPHYSEMA

The increased concentration marked "F.E." resulted from a forced expiration. The wash-out line of the slow component is drawn in. F_EHe_{s0}, the intersection of this line with the ordinate, is .0300. The values for F_EHe_{s7} and F_EHe_{s15} are .0075 and .0016, respectively.

TABLE II

FRC and other lung volumes in normal seated males

Number subjects	Age (*years*)	Surface area (M^2)	Vital capacity (*liters*)	FRC (*liters*)	Total capacity (*liters*)	Residual capacity (*liters*)	RC/TC
15	25.3 ± 2.6*	1.92 ± .14	4.80 ± .70	3.03 ± .37	6.16 ± .60	1.37 ± .34	22.3 ± 6.0

* Standard deviation.

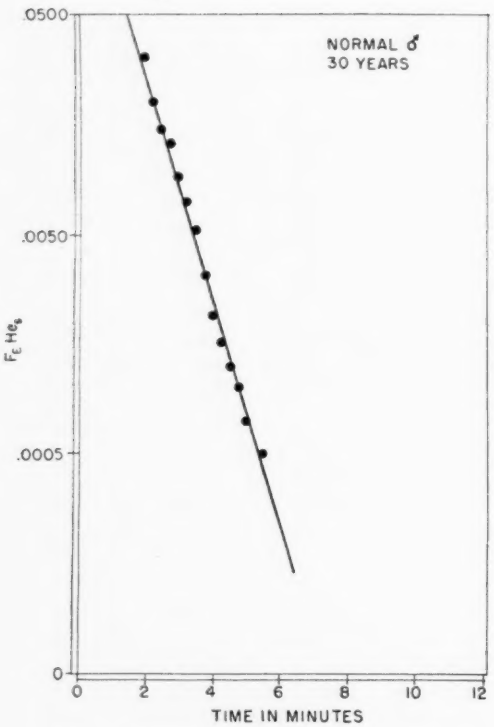

FIG. 5. NORMAL HELIUM WASH-OUT CURVE

Data relating to ventilation and lung volume in 16 normal subjects and 16 patients with pulmonary emphysema are summarized in Figure 7. Half of the normal subjects had no detectable slow space. When a slow space was found in a normal subject it comprised a relatively small fraction of the functional residual volume and had a relatively good ventilation compared to the slow spaces found in the subjects with emphysema. In the latter, the slowest spaces constituted a large part of the FRC and were very poorly aerated. In some of these subjects the greater part of the lung volume is almost without ventilation, and at least part of the remaining lung must therefore be ventilated at a much greater than normal rate.

DISCUSSION

In developing the open circuit method for estimating residual air, Darling, Cournand, and Richards (8) made allowance for nitrogen still remaining in the lungs at the end of a 7-minute wash-out period by measuring the nitrogen concentration in an alveolar air sample. This was used to represent nitrogen concentration throughout the lung, although it was well recognized that the estimate might be in error where ventilation was grossly uneven, as in severe emphysema. Such patients had 7-minute alveolar nitrogen concentrations as high as 10 or 11 per cent (9). Gilson and Hugh-Jones (10) found a concentration of 15 per cent in one subject. The present method allows an estimate of the error which may be involved in taking a 7-minute alveolar air sample to be representative of mean gas concentration in the lung. The fraction of the original quantity of helium which remains in the slow space, V_{se}, at 7 minutes is

$$\frac{F_E He_{87}}{F_E He_{s0}} = e^{-7k_s}$$

The assumption is made that nitrogen would wash out at the same rate, though it may in

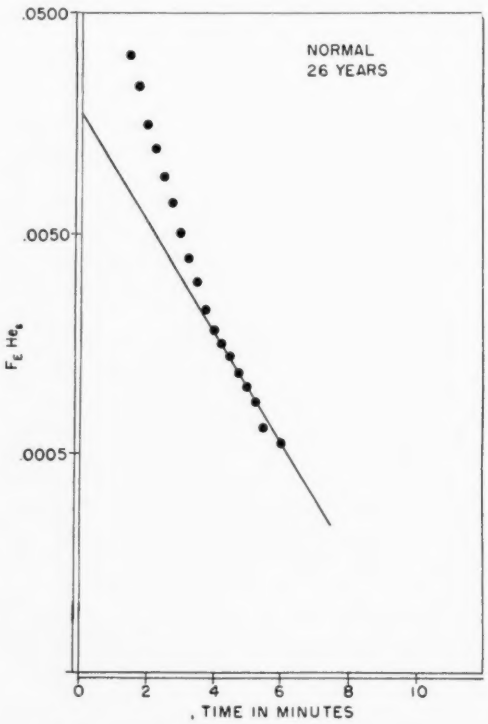

FIG. 6. NORMAL HELIUM WASH-OUT CURVE
There is a small slow space.

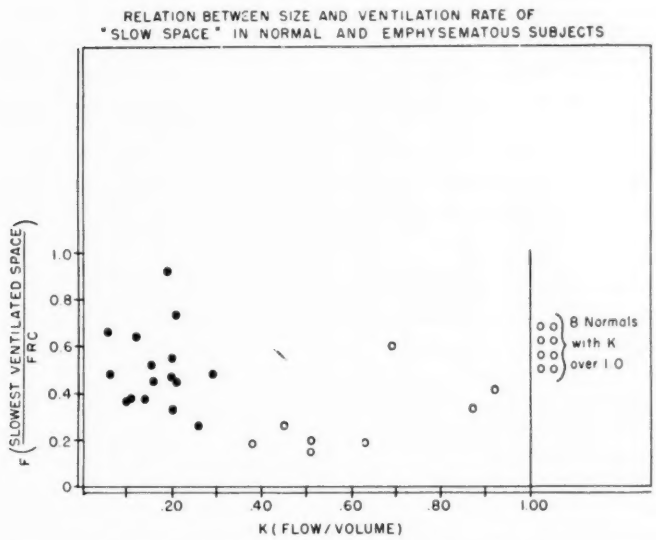

FIG. 7. SIZE AND VENTILATION RATE OF THE SLOWEST SPACE IN NORMAL PERSONS (OPEN CIRCLES) AND PATIENTS WITH EMPHYSEMA (SOLID CIRCLES)

The size of the space is expressed as the ratio between ventilation and volume of the slow space. The emphysematous subjects have relatively large and poorly ventilated slow spaces. The "normal" subject with the largest space had had bronchial asthma in childhood. Eight normal subjects have no slow space detectable by this method.

fact be much slower. Taking the starting alveolar nitrogen to be 81 per cent and assuming that nitrogen has been completely eliminated from all regions but the slowest space, the predicted mean alveolar nitrogen percentage at seven minutes would be:

$$\text{Alveolar } N_2\% = \frac{(81)(e^{-7k_s})V_{sc}}{FRC_c}$$

Applying this formula to the subjects in Table I yields the following predicted 7-minute alveolar nitrogens: C. L., 5.7 per cent; B. S., 9.9 per cent; H. B. (1), 21.3 per cent; H. B. (2), 18.9 per cent; G. H., 24.3 per cent; A. T., 16.5 per cent; and W. W., 28.6 per cent. Inspection of Figure 4 suggests that an average member of this group would have a slow component constituting about 0.5 of the FRC, with a k_s of about 0.15. The predicted 7-minute mean alveolar nitrogen would be 14.2 per cent. These high values for routinely encountered cases of pulmonary emphysema suggest that the alveolar air sampling method considerably underestimates the amount of nitrogen remaining in the lung at 7 minutes in such patients. This would result in a considerable underestimation of the FRC by the Darling method. Gilson and Hugh-Jones (10) found that the Darling method yielded lower FRC values than the closed circuit helium method in severe emphysema.

Fowler, Cornish, and Kety (11) made somewhat similar calculations of mean alveolar nitrogen in the course of their study of nitrogen wash-out and compared these with the concentration in alveolar samples. Although the concentrations in the samples were a little below the predicted values, the differences were not enough to affect seriously the Darling method. Analysis of expired nitrogen concentration during the wash-out was performed continuously by the nitrogen meter, which has so rapid a response and covers so great a concentration range that the wash-out can be followed from the very beginning. However, the analysis was abandoned when the mean expired nitrogen fell below 1 per cent, which would correspond to 0.63 per cent of helium in the present method. At this point the slowest space in most of our subjects

with greatly impaired mixing has not yet become adequately defined on the graph, and abandoning the analysis would result in missing the slowest space. In turn the amount of helium left in the lungs at 7 minutes would be very much underestimated. For the detection and measurement of these very slow spaces it is important to use an analytical method which is accurate at very low concentrations, at least when helium is used as the test gas. It seems quite possible that the same considerations may apply to nitrogen.

There is some uncertainty in predicting the behavior of nitrogen from results obtained with helium. Helium is a much more diffusible gas than nitrogen, and this should favor its wash-out rate. A slow space by the present method would probably be even slower in terms of nitrogen elimination. For this reason the helium method may underestimate to some extent the mixing inequality of the ordinary respiratory gases in severe emphysema.

The present method does not provide an overall view of intrapulmonary mixing because it cannot describe the behavior of the rapidly ventilated lung spaces. It does allow comparison of the size and ventilation rate of the worst, and sometimes next worst, ventilated regions with the total ventilation rate and FRC.

Using a closed circuit helium method, Briscoe (12) has been able to divide the lung into a well and a poorly ventilated space in cases of mixing inequality, and to calculate the ventilation rate of the slow space. Distinguishing more than one slow space by means of a closed circuit system, however, appears to involve rather complex calculation.

SUMMARY AND CONCLUSIONS

1. A convenient open-circuit method using helium is described for measuring the functional residual capacity of the lungs. After an initial period of equilibration on a helium-oxygen mixture, the subject begins to breathe oxygen, and the expired gas is collected for measurement of helium. Continuous analysis of the helium concentration in the expired gas allows calculation of the volume and ventilation rate of slowly ventilated lung spaces in patients with defective intrapulmonary gas mixing.

2. Eight of sixteen normal subjects had unequal intrapulmonary gas mixing by this method. In emphysematous subjects there were very large lung spaces with very slow ventilation rates.

3. The findings suggest that the conventional open-circuit nitrogen method may significantly underestimate the functional residual volume in patients with severely impaired intrapulmonary mixing.

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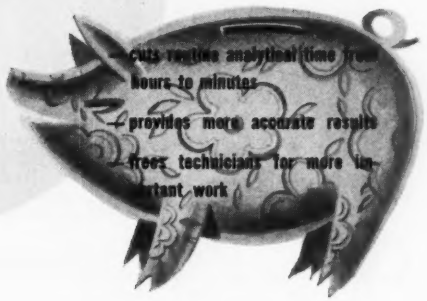
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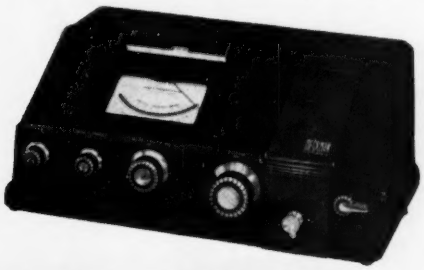

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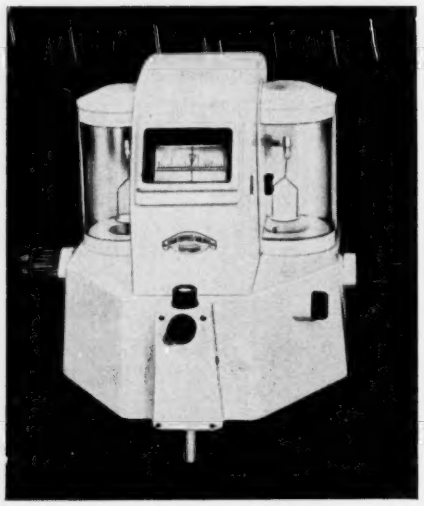

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RHEUMATIC-FEVER PROPHYLAXIS WITH BICILLIN® (DIBENZYLETHYLENEDIAMINE DIPENICILLIN G): SEATTLE STUDY

Clinical Investigation

Numerous investigations have shown that rheumatic-fever prophylaxis requires continuous control of streptococcal infection in the upper respiratory tract. Since the cost of such a long-term program may be a determining factor, studies of relatively inexpensive home therapy are of particular interest. One of these studies has been recently reported on by Tidwell,[1] speaking at the annual meeting of the American Medical Association, June, 1954.

Prophylactic medication to eradicate the beta-hemolytic streptococcus was given to 59 patients during the streptococcal season of 1952-1953. Tidwell reports that Bicillin, a highly insoluble salt of penicillin, was chosen for study because several clinical investigations had demonstrated it to be the most efficient agent for production of the extended penicillin blood levels required.

Of these 59 patients, 25 were acutely or chronically ill at the start of the program. Streptococcal pharyngitis was found in 1; congenital cardiac disease in 8; a history of rheumatic fever in 13; nephritis in 2; and asthma in 1.

THROAT CULTURES

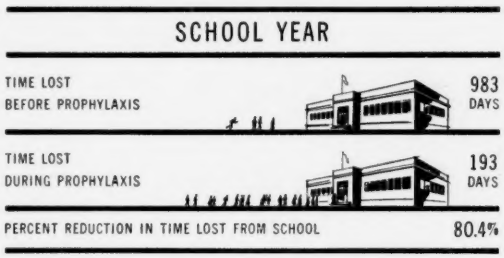
Throat cultures were performed at the beginning of the investigation, as well as monthly or oftener throughout the course. Material for culture was obtained by swabbing the tonsils and posterior pharynx through the mouth under direct vision. The swabs were then streaked on the surface of blood agar plates. These cultures were found initially positive for beta-hemolytic streptococci in 5 patients.

PROPHYLACTIC REGIMEN

One 200,000-unit tablet of Bicillin was given daily to each patient. Some patients took the medication before breakfast, others after breakfast or before lunch, others during dinner, and some at no fixed time.

RESULTS

Tidwell relates the effectiveness of the program to the patients' school attendance. During the prophylactic course, children of school age lost only 193 days because of illness, while in the year prior to the study they had lost 983 days. School absenteeism was consequently reduced by 80.4 percent.



Of all patients, only 2 showed reappearance of the beta-hemolytic streptococcus. In the first, streptococcal breakthrough appeared after the patient had been without medication for 3 days, but the culture returned to negativity when Bicillin was resumed. In the second, the culture became negative when the dose was doubled.

No early or late suppurative or non-suppurative sequelae followed any of the infections. There was neither recurrence of rheumatic fever nor evidence of subacute bacterial endocarditis.

Of the original 59 patients, 33 are being continued on a 5-year prophylactic program. The second streptococcal season has now been completed for these patients. Tidwell reports that the beta-hemolytic streptococcus has not reappeared in any of the cultures, and that there has been no recurrence of rheumatic fever or evidence of subacute bacterial endocarditis.

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